JOURNAL OF LIPID RESEARCH

VOL. 1, No. 3 APRIL, 1960

JOURNAL OF LIPID RESEARCH

D. B. ZILVERSMIT *Editor*

J. H. BRAGDON *Associate Editor* JULES HIRSCH *Associate Editor*

Editorial Board

I. L. CHAIKOFF
DON W. FAWCETT
DAVID E. GREEN
FRANK R. N. GURD
DONALD J. HANAHAN
RALPH T. HOLMAN
FORREST E. KENDALL
ROBERT G. LANGDON
S. R. LIPSKY
JAMES F. MEAD
NORMAN S. RADIN
MARVIN D. SIPERSTEIN
DANIEL STEINBERG
DANIEL SWERN

Corresponding Editors

ERICH BAER, *Canada*
SUNE BERGSTRÖM, *Sweden*
PIERRE DESNUELLE, *France*
E. KLENK, *Germany*
GEORGE POPJÁK, *England*
R. RUYSSEN, *Belgium*
B. SHAPIRO, *Israel*
MORIO YASUDO, *Japan*

Advisory Board

E. H. AHRENS, JR.
H. E. CARTER
IRVINE H. PAGE
W. H. SEBRELL, JR.

The JOURNAL OF LIPID RESEARCH is published quarterly by a nonprofit group for the promotion of basic research in the lipid field. The JOURNAL will contain original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids.

SUBSCRIPTIONS: In the United States and all foreign countries, **$6** a year. Single and back issues, when available, **$2** each. Foreign subscriptions are payable in U.S. currency, or the full equivalent. All remittances should be made payable to JOURNAL OF LIPID RESEARCH and be sent to the Business Office. Six weeks' notice is required for a change of address; the notice, to be sent to the Business Office, should give both the old and new address, including postal zone, if any.

EDITORIAL OFFICE: University of Tennessee, Memphis 3, Tennessee. (All manuscripts and editorial correspondence should be addressed to the Editorial Office.)

BUSINESS OFFICE: University Publishers Inc., 59 East 54th Street, New York 22, N. Y. Telephone: PLaza 3-6273. (All subscriptions should be addressed to the Business Office.)

Aided by United States Department of Health, Education, and Welfare, Grant HTS-5425 of the National Heart Institute.

The JOURNAL OF LIPID RESEARCH is published quarterly, in January, April, July, and October, by Lipid Research, Inc., University of Tennessee, Memphis 3, Tennessee. Subscription rate: $6.00 a year; $2.00 a single copy.

Copyright ©, 1960, by Lipid Research, Inc.
Printed in the United States of America.

JOURNAL OF LIPID RESEARCH

contents

Volume 1, Number 3
April, 1960

TITRATION OF FREE FATTY ACIDS OF PLASMA: A STUDY OF CURRENT METHODS AND A NEW MODIFICATION
David L. Trout, E. Harvey Estes, Jr., and Samuel J. Friedberg . . 199

THE STRUCTURE OF CEREBROSIDES IN GAUCHER'S DISEASE
G. V. Marinetti, T. Ford, and Elmer Stotz 203

CASTOR BEAN LIPASE: ACTION ON ITS ENDOGENOUS SUBSTRATE
Robert L. Ory, Allen J. St. Angelo, and Aaron M. Altschul . . . 208

FAT METABOLISM IN HIGHER PLANTS: XIII. PHOSPHATIDIC ACID SYNTHESIS AND DIGLYCERIDE PHOSPHOKINASE ACTIVITY IN MITOCHONDRIA FROM PEANUT COTYLEDONS
Clive Bradbeer and P. K. Stumpf 214

STUDIES ON THE DISTRIBUTION OF TOCOPHEROL IN HUMAN SERUM LIPOPROTEINS
Eugene C. McCormick, David G. Cornwell, and J. B. Brown . . . 221

PHOSPHOLIPID AND TRIGLYCERIDE METABOLISM OF EXCISED RAT DIAPHRAGM AND THE ROLE OF THESE LIPIDS IN FATTY ACID UPTAKE AND OXIDATION
E. M. Neptune, Jr., H. C. Sudduth, D. R. Foreman, and F. J. Fash . . 229

PORTAL VENOUS INJECTION OF INSULIN IN THE DIABETIC RAT: TIME OF INDUCTION OF CHANGES IN HEPATIC LIPOGENESIS, CHOLESTEROGENESIS, AND GLYCOGENESIS
Wallace R. Williams, R. Hill, and I. L. Chaikoff 236

THE TRANSPORT AND DYNAMIC STATE OF EXOGENOUS GLYCEROL- AND PALMITIC ACID-LABELED TRIPALMITIN
Raymond Reiser, Mary C. Williams, and Mary F. Sorrels 241

Notes on Methodology

APPLICATION OF A NOMOGRAM TO GRADIENT ELUTION CHROMATOGRAPHY
Huber R. Warner and William E. M. Lands 248

PREPARATION OF METHYL ESTERS
Norman S. Radin, Amiya K. Hajra, and Yukio Akahori . 250

New Methods

RECENT AND PERTINENT REFERENCES TO ANALYTICAL LIPID METHODS PUBLISHED IN OTHER JOURNALS
Compiled by Norman S. Radin, H. A. Newman, and David Kritchevsky 252

Volume 1
Number 3

Titration of free fatty acids of plasma: a study of current methods and a new modification*

David L. Trout, E. Harvey Estes, Jr., and Samuel J. Friedberg†

Central Reference Laboratory,
Veterans Administration Hospital, Durham, North Carolina,
and Department of Medicine, Duke University Medical Center,
Durham, North Carolina

[Received for publication January 9, 1960]

SUMMARY

Dole's titrimetric method for determination of the free fatty acids of plasma has been modified to improve its specificity. The fatty acid solution which is to be titrated is first washed with 0.05 per cent H_2SO_4 and thus freed of lactic acid and of an acetone-insoluble material which interferes. With this change, the Dole procedure yields results which agree well with those of Gordon's method.

Two titrimetric methods for determination of free fatty acids (FFA) of plasma, those of Dole (1) and Gordon (2), are now in wide use. Unlike the latter, the Dole method requires only moderate amounts of time, skill, and special apparatus, but it is not strictly specific for fatty acids (3). Specifically, plasma lactate interferes (2, 3), particularly at the high levels obtained with acute exercise.

A procedure was devised to wash out interfering substances and to improve the specificity of the Dole method. In testing the modified procedure, efforts were made to account for the change in apparent FFA values observed with washing.

METHODS

The Modified Procedure. It is convenient (but not essential) to use twice the volumes of plasma and of reagents specified in the Dole procedure. Thus to 2 ml. heparinized plasma in a glass-stoppered test tube are added, with shaking, 10 ml. "extraction" mixture (40 parts by volume of isopropanol, 10 parts heptane, and 1 part 1 N H_2SO_4); then 6 ml. heptane and 4 ml. water are introduced, and the mixture is shaken for at least 2 minutes. A 4- or 5-ml. aliquot of the upper, or "heptane," layer is removed to a glass-stoppered centrifuge tube and there vigorously shaken for 5 minutes with an equal volume of 0.05 per cent aqueous H_2SO_4. The tube is then centrifuged at about 300 $\times$ g for 5 minutes. To a final tube containing 1 ml. Dole's "titration" mixture is transferred 3.0 ml. of the washed heptane layer, and it is then titrated with 0.018 N NaOH while being agitated with a stream of nitrogen. The heptane layers from appropriate titration blanks and palmitic acid standards are similarly washed before titration.

Other Methods. Since lactic acid and perhaps phospholipids interfere in the Dole procedure, the concentrations of these substances were determined in washed and unwashed aliquots of heptane layer from plasma extract.

In the lactate analyses a measured volume of the heptane layer was evaporated under a stream of air at room temperature. The residue was shaken up into a water suspension, which was treated with $Ba(OH)_2$ and $ZnSO_4$ (4) and filtered. The filtrate was analyzed for lactate by the method of Barker and Summerson (5).

In the lipid phosphorus analyses the residue from the heptane layer was almost completely dissolved in alcohol-ether 3:1, and the solution filtered. Aliquots were dried and digested with hot H_2SO_4 and H_2O_2 (6). Final color development was by the method of Fiske and Subbarow (7).

* Supported in part by a research grant from the Life Insurance Medical Research Fund, in part by research Grant H-1217 (C6) from the National Heart Institute, Public Health Service, and in part by the Regional Center for the Study of Aging, Duke University.

† Research Fellow, American Heart Association.

A method was devised and tested for the evaporation of lactic acid from the heptane layer of plasma extract. The heptane layer was evaporated to near dryness, with continuous shaking, under the reduced pressure of a water aspirator. With the vacuum attachment still on, tubes were then warmed by movement in a water bath (approx. 60°C) until the last traces of solvent disappeared. The residue was dissolved in 2.0 ml. isopropanol, which was then evaporated in the same manner, after which the isopropanol treatment was repeated once.

The above procedure was tested in the following manner for its efficiency in removing lactate: Samples of the same plasmas were extracted by the Dole method with and without the addition of lactate; the heptane layers were subjected to the low-pressure treatment, reconstituted with heptane, and titrated. Titration data revealed the loss of about 90 per cent of the lactic acid originally present in the heptane layer.

Portions of lactate-depleted residue from the heptane layer of plasma extract were also largely freed of phospholipid by acetone precipitation (8). Specifically, the residue from the heptane extract from 4.0 ml. plasma was dissolved in 0.5 ml. petroleum ether (b.p. 30°-60°C); and 4.2 ml. acetone was added with shaking. Phospholipid precipitated during storage overnight with dry ice. The tube was then centrifuged, with small quantities of dry ice added to the tube carrier. One 3-ml. aliquot was titrated directly; a second portion after being washed with an equal volume of 0.05 per cent aqueous H_2SO_4.

In titrating the heptane layer residue, the solute was made up to only one-quarter to one-half the original volume, so that the sensitivity of titration was increased. In preparing titration blanks, similar volumes of heptane layer of water extracts were treated in a manner identical to those from plasma extract.

In procedures with palmitic acid-1-C^{14} the samples were added to metal planchets, evaporated to dryness under an air stream, and counted with a thin-window Geiger-Müller tube.

RESULTS

The original Dole method for FFA yields higher values than the elaborate Gordon procedure. The difference in some cases amounts to 100 to 200 μmoles per l. or up to 50 per cent. The modified Dole method, as described above, and the Gordon procedure were applied to the same 10 plasma samples. As indicated by Table 1, results by the two methods showed no significant difference.

TABLE 1. COMPARISON OF METHODS

Plasma Sample	Modified Dole Procedure	Gordon Method	Difference
	μmole/l.	μmole/l.	μmole/l.
1	271	298	−27
2	447	485	−38
3	678	731	−53
4	264	267	−3
5	258	219	+39
6	196	175	+21
7	340	359	−19
8	720	682	+38
9	1369	1373	−4
10	441	270	+171
Mean difference ± standard error			+12.5 ± 20.1

Effects of Washing Dole's Heptane Layer. It was necessary to determine whether, in washing the heptane layer, the loss of long-chain fatty acids contributed significantly to the reduction in apparent fatty acid value. Palmitic acid-1-C^{14} was used as a prototype of long-chain fatty acids and, as the sodium soap, was shaken into solution with plasma. When the labeled plasma was extracted and the heptane layer washed in the standard way, 4 per cent of the label was found in the wash solution. It was, however, also noted that heptane persisted, despite centrifugation, emulsified in the acidified water.

It was reasoned that if the radiopalmitate of wash solution existed as minute droplets of the same heptane solution which constituted the upper phase and which was to be titrated, no error would be introduced in FFA estimation. By alternate freezing in acetone and dry ice and thawing at room temperature, the emulsion was broken. After centrifugation the previously entrapped heptane was found to have the same radioisotope concentration as that removed earlier, and the wash solution retained only 0.3 per cent of the total label. It is concluded that the small loss of long-chain fatty acids in the wash procedure introduces no appreciable error.

Interference by lactic acid was also studied in the following manner: A titration blank was first prepared with a known amount of racemic lactic acid (from crystallized lithium lactate). The heptane layer was titrated and found to contain 1.5 per cent of that

acid before washing and essentially none afterward. Direct analyses of lactic acid were then made on two plasma samples and on the heptane layer obtained from the same plasmas by the original and modified Dole procedures. The data for these plasmas, as summarized in Table 2, indicate that the unwashed heptane layer of plasma extract contained at least 1.5 per cent of the lactate present and that one washing reduced this contamination by at least 80 per cent.

TABLE 2. LACTIC ACID IN PLASMA AND HEPTANE LAYERS *

	Plasma	Dole's Heptane	Washed Heptane
Subject A	1.98	0.044	.0079
Subject B	1.38	0.026	.0042

* Values are given as μmole per ml. of original plasma.

Each mg. of lactic acid per 100 ml. plasma contributed 1.5 to 2.0 μmoles per l. to the Dole FFA estimate. However, for most plasmas from resting subjects, removal of this amount of lactic acid from the heptane layer accounted for less than half of the change in titration after washing. Since it was noted that the heptane layer contained 25 to 30 per cent of the lipid phosphorus of plasma but lost nearly nine-tenths of that material with washing, it was suspected that phospholipid might interfere in the unmodified Dole method.

The total lipids (9) from two plasma samples were chromatographed on silicic acid columns, essentially by the method of Hirsch and Ahrens (10), and the phospholipid fraction was evaporated to dryness and extracted by the Dole solvent system. The heptane layer showed titratable acidity which was markedly reduced by the recommended wash procedure.

A second approach to the effect of phospholipids on the apparent FFA value was to determine the change in titration value when the heptane layer of plasma extract was freed, successively, of lactic acid and of phospholipid. The heptane layer was so treated to volatilize the lactic acid (and some fatty acid). Aliquots of the resulting residue were dissolved in heptane and titrated; other aliquots were first treated to remove phospholipid, then dissolved in heptane and titrated. Results in Table 3 show that removal of phospholipids reduces titratable acidity. In fact, after removal of both lactic acid and phospholipids, the residue yielded titration values approximating those obtained with the washed FFA method. Titration changes with washing were also pertinent; this change was greatest for the untreated heptane layer of plasma extract, less after evaporation of lactic acid, and minimal after the additional removal of phospholipids.

TABLE 3. TITRATABLE ACIDITY OF HEPTANE LAYER AFTER PARTIAL FRACTIONATION *

	Sample	Dole Procedure	After Washing
Untreated	1	.283	.235
	2	.258	.212
Vacuum-treated (to remove lactic acid)	1	.250	.229
	2	.243	.206
Vacuum-treated and freed of acetone-insoluble material	1	.239	.233
	2	.210	.200

* Values are given as μmole per ml. of original plasma.

It is concluded that an acetone-insoluble material, probably phospholipid, is extracted and titrated in the Dole method but is largely washed out in the modified procedure.

Recovery of Added Fatty Acids. The chief components of plasma FFA, sodium palmitate, oleate, and stearate (11), were dissolved in plasma, and their recovery by the modified Dole procedure was determined. Specifically, 1.2 μmoles of each soap was added to one of three 2.0-ml. aliquots of plasma; these and a fourth unsupplemented portion were analyzed for FFA. This set of four analyses was carried out on each of ten plasmas.

Results, as shown in Table 4, indicate that recoveries of each of the three fatty acids studied did not differ significantly and that the mean recoveries exceeded 90 per cent.

TABLE 4. RECOVERIES OF FATTY ACIDS ADDED TO PLASMAS

Fatty Acid	Number of Trials	Recoveries *
		per cent
Palmitate	10	93.6 ± 0.69
Oleate	10	93.5 ± 0.85
Stearate	10	92.8 ± 0.94

* Mean ± standard error.

Reproducibility of the Modified Dole Method. Reproducibility of the original and modified Dole procedures was determined and compared in terms of standard deviation of repeated analyses. A total of 38 analyses was carried out by each method on the same 12 plasma samples. By Dole's original method, a mean FFA of 689 μmoles per l. with a standard deviation of 11.9 was found; the modified method gave 584 ± 11.2 μmoles per l.

DISCUSSION

The modified Dole procedure, as described above, preserves most of the ease and reproducibility of the original Dole FFA method but greatly improves its specificity for fatty acids. In the original Dole procedure an acetone-insoluble lipid, probably a phospholipid, interferes significantly. Lactic acid of normal fasting plasma also brings about an error of several per cent, and this error increases as the lactic acid rises with exercise, psychological stress, diabetes, or infusion of epinephrine or norepinephrine. The recommended wash treatment minimizes interference by these two materials.

We are grateful for technical assistance from Miss Helen L. Hilderman, Mrs. Mary Ruth Greenfield, and Mrs. Nancy C. Johnson.

REFERENCES

1. Dole, V. P. *J. Clin. Invest.* **35**: 150, 1956.
2. Gordon, R. S., Jr. *J. Clin. Invest.* **36**: 810, 1957.
3. Fredrickson, D. S., and R. S. Gordon, Jr. *Physiol. Revs.* **38**: 585, 1958.
4. Somogyi, M. *J. Biol. Chem.* **160**: 69, 1945.
5. Barker, S. B., and W. H. Summerson. *J. Biol. Chem.* **138**: 535, 1941.
6. Youngburg, G. E., and M. V. Youngburg. *J. Lab. Clin. Med.* **16**: 158, 1930.
7. Fiske, C. H., and Y. Subbarow. *J. Biol. Chem.* **66**: 375, 1925.
8. Hanahan, D. J., J. C. Dittmer and E. Warashina. *J. Biol. Chem.* **228**: 685, 1957.
9. Lipsky, S. R., A. Haavik, C. L. Hopper, and R. W. McDivitt. *J. Clin. Invest.* **36**: 233, 1957.
10. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.
11. Dole, V. P. *A.M.A. Arch. Internal Med.* **101**: 1005, 1958.

Volume 1
Number 3

The structure of cerebrosides in Gaucher's disease*

G. V. Marinetti, T. Ford, and Elmer Stotz

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester 20, N. Y.

[Received for publication October 24, 1959]

SUMMARY

Crystalline cerebrosides were isolated from the spleen of a woman with Gaucher's disease. The cerebrosides accounted for 38 per cent of the total spleen lipids and 0.89 per cent of the fresh spleen. The sugar moiety of the cerebrosides was found to be glucose and its position of attachment was established to be on the primary hydroxyl group of sphingosine. The double bond in the sphingosine moiety had the *trans* configuration. The fatty acids of the cerebroside were determined by gas chromatography and paper chromatography and found to be mainly lignoceric, behenic, and palmitic acid. Small amounts of arachidic and stearic acids were also present.

Cerebrosides isolated from persons with Gaucher's disease contain predominantly glucose (1 to 5) in place of galactose. Carter and Greenwood (6) have shown that in the case of phrenosin the galactose is attached to the primary hydroxyl group of sphingosine. However, the position of attachment of glucose in the abnormal Gaucher's cerebroside from humans has not been investigated. In addition, the nature of the fatty acids of the Gaucher's cerebrosides has not been sufficiently studied. This paper will elucidate both of these points.

METHODS

A portion of human spleen (310 g. wet weight of a 780 g. spleen) was obtained from a woman (age 50) with Gaucher's disease immediately after surgery. The spleen was minced, washed with isotonic saline, and then extracted with methanol-ether 1:1 (v/v) in the usual way. The yield of total lipid was 7.26 g.

Column chromatography of the spleen lipids was carried out on Florisil[1] (7) and on silicic acid (8). The cerebrosides isolated by chromatography on Florisil were recrystallized several times from acetic acid. Hydrolysis, acetylation, reductive hydrogenolysis, and isolation of diacetylsphingine were carried out by procedures similar to those described by Carter and Greenwood (6). Nitrogen and phosphorus analyses were carried out as described previously (8). Sugar was analyzed by the method of Radin *et al.* (9). Carbon, hydrogen, and acetyl group analyses were done by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

The cerebrosides were hydrolyzed with aqueous HCl by the procedure of Rosenberg and Chargaff (5) and the osazone derivative prepared in the usual manner (10). Paper chromatography of the sugars was carried out by the ascending technique in three solvent systems: (*a*) *n*-butanol-pyridine-water 3:1:1.5 (v/v/v) (upper phase of this mixture was mixed with 1 volume of pyridine); (*b*) ethyl acetate-pyridine-water 30:21:9 (v/v/v); and (*c*) isopropyl ether-formic acid 3:2 (v/v).

The fatty acid methyl esters were analyzed by gas chromatography. The analysis was kindly performed by Dr. M. Kates, of the National Research Council, Ottawa, Canada. The free fatty acids were analyzed by paper chromatography on mineral oil-impregnated paper (11). The solvent system consisted of acetic acid-water 195:5 (v/v) (saturated with mineral oil). Whatman No. 1 filter paper was impregnated by dipping into a 10 per cent (v/v) solution of mineral oil in benzene.

Paper chromatography of diacetylsphingine was carried out on silicic acid-impregnated paper, employing a solvent of *n*-hexane-diisobutylketone 70:60 (v/v) (12).

Infrared spectral analyses were kindly run by Dr. W. B. Mason and Mr. A. Behringer, of the Atomic Energy Project of the University of Rochester, and were made possible by funds from the Atomic Energy Commission. A Perkin-Elmer Model 21 instrument

* Aided in part by United States Public Health Service Grant H-2063 of the National Heart Institute.

[1] Floridin Co., Tallahassee, Fla.

with NaCl prism was used. The spectra were run on pressed KBr disks at a concentration of 5 μg. of compound per 1 mg. of KBr. The compounds were incorporated into the KBr by the lyophilization technique (13).

RESULTS

The lipid content of the spleen on a wet weight basis was 2.35 per cent. The phosphorus content of the lipids was 1.86 per cent. Thirty-eight per cent of the total spleen lipids (or 0.89 per cent of the wet weight spleen) was cerebroside. The nonphosphatides (triglycerides plus total cholesterol) as determined by column fractionation on silicic acid constituted 17 per cent of the total lipids.

The cerebrosides (2.74 g.) obtained by column chromatography on Florisil were found to contain 0.13 g. of phosphatide (determined by P analysis). Three recrystallizations from acetic acid yielded 1.7 g. of crystalline cerebrosides (I) (m.p. 169°-171°C begins to sinter at 80°C). The analysis of cerebroside (I) was as follows: 69.35 per cent C; 11.55 per cent H; 1.76 per cent N; 23.7 per cent glucose. (Calculated values for $C_{46}H_{89}O_8N$ [784.2]: 70.50 per cent C; 11.42 per cent H; 1.78 per cent N; and 23.0 per cent glucose.) Cerebrosides (I) were free from P.

TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACIDS OF THE SPLEEN CEREBROSIDES (I)

	moles per cent
C_{24} (lignoceric acid)	46
C_{22} (behenic acid)	27
C_{20} (arachidic acid)	3
C_{18} (stearic acid)	3
C_{16} (palmitic acid)	20

The acetic acid supernatant fluids from the recrystallized (I) were found to contain cerebrosides and phosphatides. Hydrolysis of this fraction showed glucose to be the only sugar present. The fatty acids on these cerebrosides were not analyzed because of the contaminating phosphatide.

Attempts to isolate and purify the cerebrosides by chromatography on silicic acid were unsuccessful. The cerebrosides were eluted with 20 per cent methanol in chloroform along with phosphatidylethanolamine and phosphatidylserine.

The fatty acid methyl esters obtained by hydrolysis of the cerebrosides with methanolic-sulfuric acid were analyzed by gas chromatography. The results are given in Table 1. It is evident that the major components are lignoceric, behenic, and palmitic acid. The fatty acids and sphingosine were isolated in nearly theoretical yields.

The fatty acid methyl esters were converted to the free fatty acids which were then analyzed by paper chromatography (11). The major acids which were observed were lignoceric, behenic, and palmitic. Stearic and arachidic acids were barely detectable on the chromatograms. Hence the paper chromatographic findings are in agreement with the gas chromatographic analyses. The acids were saturated since they gave an essentially negative test with permanganate. Since the cerebroside (I) yielded a pentacetyl derivative rather than a hexacetyl derivative, the presence of an appreciable amount of hydroxy acids is ruled out.

Analytical data on the acetylated (II) and the re-

TABLE 2. ANALYTICAL DATA ON THE ACETYLATED CEREBROSIDES

	N	Glucose	Acetyl	$\frac{\text{Glucose}}{\text{N}}$	$\frac{\text{Acetyl}}{\text{N}}$
	per cent	*per cent*	*per cent*		
Calculated for pentacetyl compound $C_{56}H_{99}O_{13}N$ (994.4)	1.41	18.2	21.6	1.0	5.0
Found: Compound (II) m.p. 84°-87°C	1.41	18.2	21.2	1.0	4.93
Calculated for reduced tetracetyl compound $C_{54}H_{99}O_{11}N$ (938.4)	1.49	19.2	18.3	1.0	4.0
Found: Compound (III) m.p. 94°-96°C	1.46	18.5	19.3	0.99	4.3

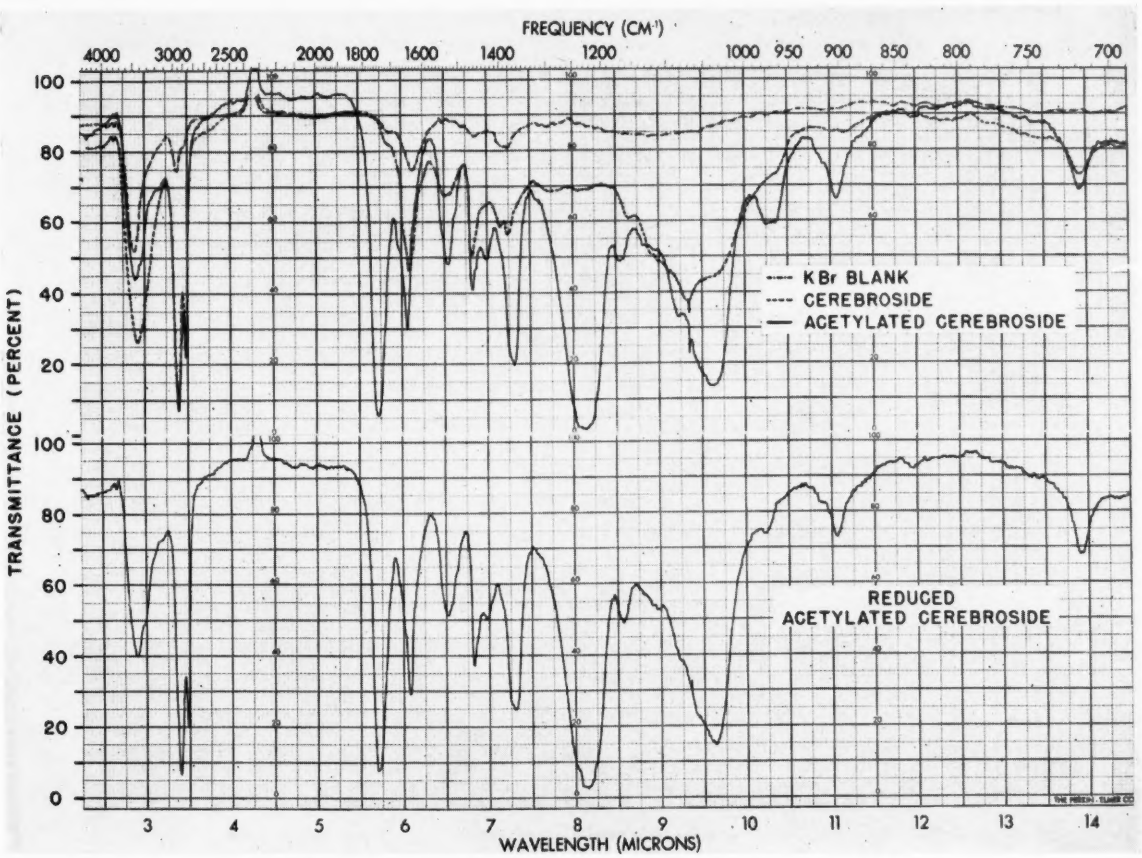

FREQUENCY (CM⁻¹)
4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
TRANSMITTANCE (PERCENT)
100 80 60 40 20 0
KBr BLANK
CEREBROSIDE
ACETYLATED CEREBROSIDE
REDUCED
ACETYLATED CEREBROSIDE
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

FIG. 1. Infrared spectra of cerebrosides (I), acetylated cerebrosides (II), and reduced acetylated cerebrosides (III). The KBr blank is also shown.

duced acetylated cerebrosides (III) are given in Table 2. In our hands the extent of hydrogenolysis was 84 per cent. (Carter and Greenwood [6] found 40 per cent with phrenosin.) No sugar was liberated during the reduction with hydrogen. The data in Table 2 demonstrate that the acetylated cerebroside (II) was a pentacetyl derivative, but that the reduced derivative (III) was predominantly a tetracetyl derivative but did contain some pentacetyl compound. Hence it is clear that approximately one acetyl group was lost during hydrogenolysis. The reduced compound (III) was a mixture of tetracetyl and pentacetyl derivatives because the extent of hydrogenolysis was 84 per cent.

TABLE 3. PROPERTIES OF DIACETYLSPHINGINE

	N	Melting Point	R_f Value
	per cent		
Calculated: $C_{22}H_{43}O_3N$ (369.6)	3.80	105°–106°C*	0.45*
Found: Compound (IV)	3.89	100°–104°C	0.45

* Melting point and R_f value of the authentic compound.

Hydrolysis of the reduced acetylated cerebrosides (III) (150 mg.) yielded sphingine and some dihydrosphingosine. These were converted to the corresponding diacetylsphingine (IV) and triacetyldihydrosphingosine (V). Crystallization from ethanol yielded 30 mg. of crystalline diacetylsphingine in about 95 per cent purity. (The theoretical yield based on 84 per cent hydrogenolysis would be 39 mg.) Analytical data and properties of the diacetylsphingine (IV) are given in Table 3. The infrared spectra of compounds I to IV and of the authentic sample of diacetylsphingine (kindly donated by Dr. H. E. Carter, of the University of Illinois) are shown in Figures 1 and 2. The

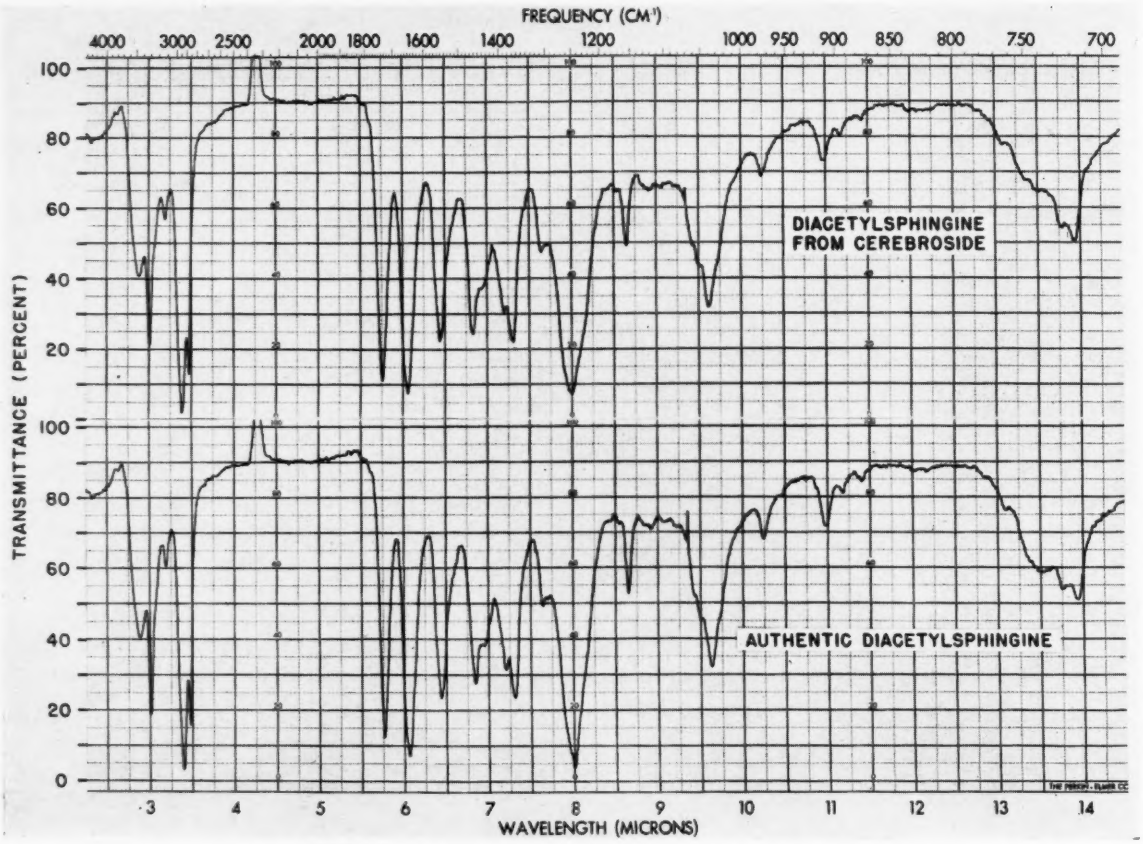

FREQUENCY (CM⁻¹)
4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
TRANSMITTANCE (PERCENT)
100 80 60 40 20 0
DIACETYLSPHINGINE FROM CEREBROSIDE
AUTHENTIC DIACETYLSPHINGINE
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

FIG. 2. Infrared spectra of diacetylsphingine (IV), which was isolated from the Gaucher's cerebroside, and of the authentic diacetylsphingine.

spectra of the two compounds are essentially identical. The slightly lower melting point of compound (IV) as compared to the authentic diacetylsphingine was due to the presence of a very small amount of the triacetyldihydrosphingosine (V) (m.p. 100°-102°C) (14).

The diacetylsphingine from the Gaucher's cerebroside had the same mobility as the authentic diacetylsphingine when chromatographed on silicic acid-impregnated paper. The small amount of triacetyldihydrosphingosine in compound (IV) was visible on the chromatograms. These compounds were detected by staining with Rhodamine 6G.

The cerebroside (I) (100 mg.) was hydrolyzed for 90 minutes in 3N aqueous HCl. After extraction of the fatty acids with ether, the aqueous phase was analyzed for sugar by paper chromatography and the osazone derivative was then prepared. The sugar in the spleen cerebrosides migrated exactly as glucose in three different solvents and yielded an osazone which had the same crystal structure as glucosazone.

Galactose was not detected on the chromatograms. However, if 1 to 5 per cent galactose were present, it is doubtful if this small amount could be detected. It is clear then that the presence of small amounts of galactocerebrosides cannot be ruled out. The enzymatic assay for glucose as performed by Rosenberg and Chargaff (5) was not carried out. This enzymatic assay is quite specific for glucose but again it is doubtful whether it could rule out the presence of very small amounts of galactose.

DISCUSSION

Normal cerebrosides contain galactose as the major sugar, whereas cerebrosides in Gaucher's disease contain predominantly glucose (1 to 5). Although Carter and Greenwood (6) have shown that in the case of phrenosin the galactose is linked to the primary hydroxyl group of sphingosine, there are no experimental data to demonstrate that the glucose in the abnormal

cerebroside is attached to the same position. In view of our observation that galactocerebrosides are hydrolyzed more rapidly with acid than the glucocerebrosides, it seemed important to examine the structure of the Gaucher's cerebroside. Such a study would more fully elucidate the nature of the molecular defect in Gaucher's disease. In addition to the sugar component, a study of the nature of the fatty acids and the configuration of the double bond in sphingosine of the Gaucher's cerebroside was carried out.

The result of these studies conclusively shows that the major sugar in this particular Gaucher's cerebroside is glucose and that it is attached to the primary hydroxyl group of sphingosine. Furthermore, the double bond in the sphingosine moiety has the *trans* configuration, since on reduction the band at 10.3 μ. is markedly diminished (Fig. 1). These structural features are identical to those of the galactocerebroside phrenosin (6, 15) and sphingosine (16, 17).

With regard to the fatty acids on the cerebrosides, this study for the first time gives a detailed analysis of these constituents in Gaucher's disease. It is clear that the cerebrosides are heterogeneous with respect to the fatty acids and are to be considered as a family of compounds. The major cerebrosides in this case are lignoceryl, behenyl, and palmityl derivatives. The stearyl and arachidyl derivatives are minor components. How these acids compare to the acids in normal human spleen cerebrosides cannot be ascertained at the present time since similar detailed studies have not been carried out on normal persons. In our hands attempts to isolate a sufficient amount of pure cerebrosides from the spleens of five normal humans have been unsuccessful because of the small amount of cerebroside which is present.

Recently Rosenberg and Chargaff (5) have reported that a cerebroside isolated from a person with Gaucher's disease might be a behenyl derivative. However, their assumption was based on elementary analysis and on the yield of mixed fatty acid esters. These data cannot distinguish the type of fatty acids present but only give the average chain length of the mixture. In our case, and very likely in theirs, the average fatty acid chain length was 21 to 22 carbon atoms.

The elucidation of molecular diseases must in the final analysis depend on precise chemical data. The work reported here is aimed toward this end.

REFERENCES

1. Klenk, E., and R. Harle. *Z. physiol. Chem. Hoppe-Seyler's* **189**: 243, 1930.
2. Halliday, N., H. J. Deuel, Jr., L. J. Tragerman, and W. E. Ward. *J. Biol. Chem.* **132**: 171, 1940.
3. Klenk, E., and F. Rennkamp. *Z. physiol. Chem. Hoppe-Seyler's* **273**: 253, 1942.
4. Devor, A. W. *Arch. Biochem. Biophys.* **50**: 217, 1954.
5. Rosenberg, A., and E. Chargaff. *J. Biol. Chem.* **233**: 1323, 1958.
6. Carter, H. E., and F. L. Greenwood. *J. Biol. Chem.* **199**: 283, 1952.
7. Radin, N. S., J. R. Brown and F. B. Lavin. *J. Biol. Chem.* **219**: 977, 1956.
8. Marinetti, G. V., J. Erbland and J. Kochen. *Federation Proc.* **16**: 837, 1957.
9. Radin, N. S., F. B. Lavin and J. R. Brown. *J. Biol. Chem.* **217**: 789, 1955.
10. Schreiner, R. L., and R. C. Fuson. *The Systematic Identification of Organic Compounds,* 3d ed., New York, John Wiley & Sons, Inc., 1948, pp. 116–17.
11. Kaufman, H. P., and W. H. Nitsch. *Fette, Seifen, Anstrichmittel* **56**: 154, 1954.
12. Marinetti, G. V., J. Erbland and E. Stotz. *J. Am. Chem. Soc.* **80**: 1624, 1958.
13. Mason, W. B. *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy,* March, 1958.
14. Carter, H. E., and Y. Fujino. *J. Biol. Chem.* **221**: 879, 1956.
15. Marinetti, G., and E. Stotz. *J. Am. Chem. Soc.* **76**: 1347, 1954.
16. Mislow, K. *J. Am. Chem. Soc.* **74**: 5155, 1952.
17. Fodor, G., and J. Kiss. *Nature* **171**: 651, 1953.

J. Lipid Research
April, 1960

Castor bean lipase: action on its endogenous substrate*

ROBERT L. ORY, ALLEN J. ST. ANGELO, and AARON M. ALTSCHUL

Southern Regional Research Laboratory,
Southern Utilization Research and Development Division,
Agricultural Research Service, U. S. Department of Agriculture,
New Orleans 19, Louisiana

[Received for publication November 24, 1959]

SUMMARY

A method is described for obtaining a lipase emulsion from castor beans which rapidly hydrolyzes all of its endogenous substrate at an optimum pH of 4.0 to 4.2. The course of hydrolysis can be described by a first-order reaction; no additives are required for activity and the reaction goes to completion in less than 1 hour. The enzyme emulsion (oil-in-water) is stable for at least 2 weeks in a pH 7.0 phosphate buffer containing added cysteine and ethylenediaminetetraacetic acid; it is rather heat stable, but the activity is lowered by mechanical shaking. Reagents that react with sulfhydryl groups inactivate the enzyme; the inhibition by mercuric ion and *p*-chloromercuribenzoic acid can be reversed with an excess of cysteine. The lipase emulsion is not inhibited by diisopropylfluorophosphate in concentrations lower than 0.01 M, nor by protamine sulfate and heparin in relatively high concentrations. It is inhibited by 0.01 M fluoride.

Although there is an extensive literature on the action of seed lipases on their own substrates *in situ* (1, 2, 3) and of partially purified enzymes on a variety of substrates (4), there is no information on the behavior of the partially purified lipases on their own natural substrates. In most instances the source of lipase was oil-free tissue (5), but even when the original tissue was used, an excess of additional substrate was added; it is the hydrolysis of this added substrate which was followed.

Seed lipases might well be studied by starting first with crude systems and endogenous substrates. Thereafter any modifications in the enzyme system during purification can be detected by comparison of its behavior with that of the crude enzyme on its endogenous substrate. The lipase in castor beans *(Ricinus communis)* is particularly suitable for study of the crude system since this enzyme is reported to have its optimum activity at acid pH. Hence the operations of isolation of the enzyme and substrate can be carried out at neutral pH with no hydrolysis taking place.

One of the difficulties in studying this enzyme has been the presence of allergens in the pomace (6, 7). Instances are known in which investigators became allergic to this material and were required to terminate their research on the lipase at an early stage. It is possible to minimize this problem by extraction of the kernel with water or buffer followed by high speed centrifugation: the oil and a small amount of protein float to the top and the bulk of the proteins remain in the underlayer which is discarded. The fatty layer contains the lipase: this can easily be demonstrated by adjusting the pH to around 4, whereupon there is rapid and complete hydrolysis of the oil.

This is a report of the properties of the crude lipase of castor bean acting on its own endogenous substrate. The hydrolysis can be described as a first-order reaction throughout the range of substrate concentration with a maximum rate between pH 4 and 4.2. The enzyme in the presence of substrate is relatively heat stable; it is inhibited by reagents which react with sulfhydryl groups. Adjustment of pH is all that is necessary for activation of the enzyme; no additional calcium ion, other salts, or serum albumin are required for activity.

MATERIAL AND METHODS

Seed. Castor beans *(Ricinus communis)*, Cimmarron variety, were used unless otherwise indicated. The seeds were two years old when obtained; they were stored at 15°C until used.

* Presented in part at the 134th National Meeting, American Chemical Society, Chicago, September 7–12, 1958.

Preparation of Lipase Emulsion. The castor beans were soaked in water overnight at 4°C; all subsequent operations in preparing the lipase emulsions were also carried out at this temperature. The swollen seeds, containing about 20 per cent water, were dehulled, ground with sand in 20 ml. of extraction medium (0.05 M cysteine, 0.1 M phosphate buffer, pH 7.0, 0.01 M EDTA[1] per 20 to 25 g. of beans), and filtered through cheesecloth. This operation was repeated twice; the extracts were combined and centrifuged at 11,000 × *g* for 30 minutes. The thick fatty layer was removed by spatula, mixed with 2 volumes of the same buffer, and stored at 4°C until used. These lipase emulsions contain about 24 per cent lipid and 1 per cent protein. It is interesting to note that the ratio of protein to fat is 0.04, which is in the same range as that reported for chylomicrons (8).

Measurement of Activity. Two ml. of the lipase emulsion and 4 ml. of water were stirred by magnetic stirrer for 10 minutes at room temperature (24°C) in order to bring the test mixture into temperature equilibrium. This treatment did not impair the enzyme activity. An aliquot was withdrawn for analysis of initial ester (glycerides) content and 4 ml. of buffer at the same temperature was added to adjust to pH 4 (unless stated otherwise), thus starting the reaction. Samples for analysis were withdrawn at stated intervals and were pipetted into 10 ml. alcohol-ether (1:3, v/v), which was then boiled to coagulate the protein. These solutions were filtered through sintered glass to remove precipitated protein and evaporated on a steam cone to dryness. The dry fat was then taken up in 15 ml. of dry peroxide-free ether (the ether was always freshly purified by passage over a column of activated alumina), 0.3 ml. each of 2.5 per cent solutions in alcohol of hydroxylamine hydrochloride and sodium hydroxide were added, and the flasks placed in a water bath at 60°C until the ether was evaporated, and then held there for an additional 10 seconds to complete hydroxamate formation. Five ml. of ferric perchlorate (0.32 per cent in alcohol, prepared fresh daily) was added and, after waiting at least 20 minutes for maximum color development, the absorption of the samples was read in a spectrophotometer at 520 mμ.

This procedure for ester content is based on the formation of a ferric hydroxamate-fatty acid complex (9, 10). Methyl ricinoleate was the standard used for calibration of the analytical procedure.

[1] The following abbreviations are used in the text: EDTA, ethylenediaminetetraacetic acid; NEM, N-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; and DFP, diisopropylfluorophosphate.

The relative rate of hydrolysis was obtained from the first-order rate constant as is described in the next section.

RESULTS

Rate of Hydrolysis. The course of hydrolysis in a typical reaction is given by the experimental points in Figure 1. When the logarithm of the ester concentration is plotted against time elapsed from the start of the reaction, the experimental points fall on a straight line (*A*, Fig. 1). The relative lipase activity (k/2.303) is given by the first-order rate constant, $\frac{k}{2.303} = \frac{\log S_o - \log S_t}{t}$, where S_o is the original ester concentration and S_t is the ester concentration at time, t; the initial rate of hydrolysis may be calculated by multiplying the rate constant by the initial ester concentration. For the data in Figure 1 the initial rate is 6.2 mg. of ester per ml. per minute. Line *B* (Fig. 1) was calculated from the rate constant obtained from the data in curve *A* (Fig. 1); the open circles are the experimental points.

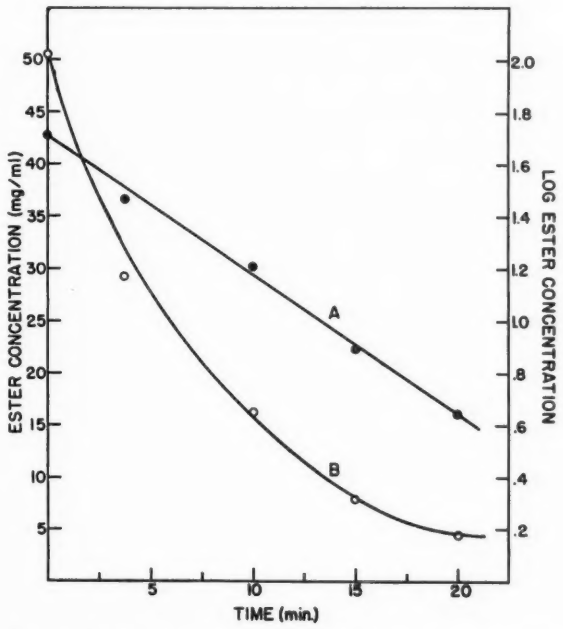


FIG. 1. Hydrolysis of endogenous castor oil by crude castor lipase at pH 4.5 in acetic acid-sodium acetate buffer. Reaction flask contained 2 ml. lipase emulsion, 4 ml. water, and 4 ml. buffer. The scale at the left is for curve *B*.

From these data it is evident that hydrolysis in this system is rapid and complete; no added fatty acid acceptor is needed, nor was there any evidence of reversal of hydrolysis. The course of hydrolysis is described by first-order kinetics over practically the entire range of substrate concentration. Such behavior differs from hydrolysis of fats by pancreatic lipase in which the relative rate of the reactions sharply decreases in the order: triglyceride → diglyceride; diglyceride → monoglyceride; and monoglyceride → glycerol (11). It is possible that the rate-limiting reaction in the hydrolysis by castor bean lipase is the step: triglyceride → diglyceride. Under such circumstances the reaction would have the same type of kinetics throughout the entire range of substrate concentration.

pH Optimum. The effect of pH on the relative rate of hydrolysis was obtained by using a number of different acetate and phosphate buffers. The optimum pH is in the range of 4.0 to 4.2. This is a little lower but in general agreement with the work of previous investigators who listed the optimum pH to be in the range of 4.5 to 5.0 (12, 13, 14).

Stability. The problem of stability in a system containing both enzyme and substrate must take into account both the hydrolysis of the substrate prior to testing and the stability of the enzyme. A comparison was made of the stability of substrate in two different media used for extraction and preparation of the lipase emulsion. Hydrolysis of endogenous substrate was rather rapid in an unbuffered medium adjusted initially to pH 7 with sodium hydroxide; the ester content was reduced from 22 to 5 mg. per ml. in 6 days. The substrate was stable for over a week in a cysteine-sodium hydroxide buffer.

The enzyme activity is stable in buffered media for at least 14 days; there does not seem to be any advantage in adding cysteine to a buffered solution. The combination of phosphate buffer (0.1 M, pH 7), cysteine (0.05 M), and EDTA (0.01 M), however, gave a somewhat higher rate; hence this was used as the standard medium in preparing the lipase emulsions.

The inclusion of cysteine was suggested by reports that some other plant lipases (15) are sensitive to sulfhydryl reagents and are stabilized by cysteine. The combination of EDTA and cysteine was prompted by its successful use in stabilizing papain (16).

Mechanical Stability. Each determination of lipase activity in these experiments was made on a separate vial of lipase emulsion which was stored undisturbed until assayed. This same stability was not observed when, in the course of experiments on successive days, aliquots of lipase emulsion were removed from a single preparation kept in a stoppered glass cylinder and shaken well before withdrawal of each aliquot. This suggested that the lipase was unstable to even this small amount of shaking. There was also the possibility that the renewal of oxygen which took place when the aliquot was withdrawn was a factor in reduction of activity.

To test these points, a freshly prepared standard lipase emulsion was divided into three parts: One-third was distributed as 2 ml. portions in 5 ml. capacity vials, stoppered, and stored at 4°C without further shaking; one-third was distributed into similar vials and placed on a slowly rocking apparatus at 4°C for continuous agitation; the remaining third was retained in the graduated cylinder at 4°C and shaken and opened twice or more daily to simulate normal sampling procedure. The results showed conclusively that the enzyme preparation is sensitive to shaking with or without renewal of the air above the emulsion. The enzyme in vials which were undisturbed retained activity for 18 days, whereas the other two groups kept for the same length of time decreased in activity 46 and 61 per cent, respectively. In view of this finding, samples from freshly prepared lipase emulsions were distributed into separate vials and stored undisturbed until tested.

Stability to Heating. Flasks containing the castor bean lipase emulsion, still at pH 7.0, were set in water baths at designated temperatures and time intervals, then immediately immersed in ice baths to reduce the temperature quickly. Afterward the flasks were placed on a magnetic stirrer, the buffer was added to initiate the reaction, and the rate of hydrolysis was measured in the usual way. The effect of length of heating at various temperatures on inactivation of the enzyme is shown graphically in Figure 2. It is noteworthy that

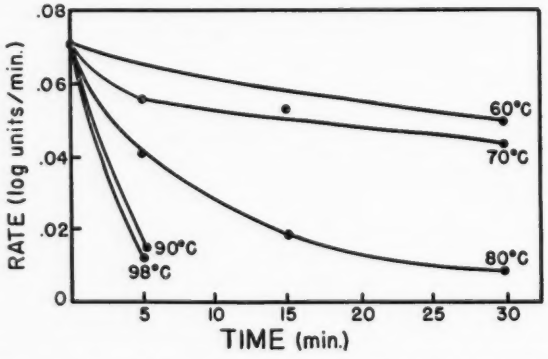


FIG. 2. Heat stability of crude castor lipase emulsion. The abscissa indicates the length of time heated at each temperature.

the castor lipase in the fatty layer seems rather heat stable: only about 20 per cent of the activity was lost by heating at 60°C for 30 minutes at pH 7.0. No activity was lost on standing for 60 minutes at 24°C.

Effect on Activity of Soaking of Castor Beans. Since some seeds, e.g., peanut and cottonseed (17), exhibit little or no lipolytic activity *in vitro* but assume such activity on germination, the question arose whether the presoaking of the castor beans in water even at 4°C might be inducing germination with a subsequent increase in lipase activity. Lipase emulsions prepared from presoaked and unsoaked beans were tested for activity in the usual way. There was little difference in relative activity, the rates being 0.092 and 0.076 log units per minute, respectively. Presoaked beans were much easier to dehull; hence presoaking was included as part of the standard procedure.

Effect of Variety and Age of Seed. There is a decrease in lipase activity of castor beans on long storage: the relative activity of the enzyme from two-year-old beans was 0.064 log units per minute, and after another year of storage of the beans, it was 0.036. A fresh sample of castor beans (less than one year old), Baker 296 Inbred variety, had a relative enzyme activity of 0.065 log units per minute.

Effect of Metal Ions. Various metals, as the acetate salts, adjusted to pH 6.0 to 7.0, were incubated with the lipase emulsion containing no cysteine or EDTA for 10 minutes. Then the pH was lowered and the activity measured with the results as summarized in Table 1. Ferric iron, zinc, and magnesium ions in the concentrations listed had no effect on the enzyme. Calcium ion inhibited but slightly at the high concentration. It accelerated the reaction at lower concentrations but not to the extent reported for other lipases, for example, pancreatic lipase (18). Copper ion (Cu^{2+}) inhibited at the higher concentration; lead produced slight inhibition. The most powerful inhibitor of the group tested was mercuric ion, which produced 67 per cent inhibition at a concentration of 6×10^{-5} M.

Effect of Additives. The influence of various other additives on the activity of the lipase emulsion was studied in a similar manner with results as given in Table 2. Serum albumin, which acts as a fatty acid acceptor for lipoprotein lipase (19), had no effect on the rate or course of hydrolysis over a wide range of concentration. Though it had no effect on the rate of reaction, serum albumin did have a visible effect on the appearance of the emulsion; when it was present in the reaction mixture, the emulsion appeared to be more stable physically. Protamine and sodium heparinate inhibit lipoprotein lipase of adipose tissue (20), but neither had any influence on the activity of this enzyme.

Sodium fluoride (0.01 M) inhibited the activity of castor bean lipase. This behavior is in accord with the reported inhibition of other lipases by fluoride (21, 22). Cyanide did not inhibit in the concentration used.

PCMB produced complete inhibition at 0.001 M concentration. Inhibition by NEM, much less potent in this instance than PCMB, seemed to depend upon the time of incubation. Cysteine completely reversed the inhibition by PCMB; it also reversed inhibition by mercuric ion. This evidence is suggestive that at least one —SH group is involved in the functional activity of the lipase.

DFP, a powerful inhibitor of some hydrolases, does not inhibit pancreatic lipase and in some concentrations even appears to stabilize the enzyme (23), presumably protecting it from destruction by other enzymes present in the crude preparation. DFP inhibited the castor bean lipase emulsion, but only incompletely and in much higher concentrations than needed normally; 0.02 M and higher as compared to 10^{-6} M and lower reported for other enzymes.

TABLE 1. EFFECT OF METAL IONS ON HYDROLYSIS OF ENDOGENOUS SUBSTRATE BY LIPASE EMULSION *

Metal	Concentration	Relative Activity
	M	*Per cent*
None		100
Ca^{++}	1×10^{-2}	76
	6×10^{-3}	100
	1×10^{-3}	137
	6×10^{-4}	124
Zn^{++}	6×10^{-4}	99
Fe^{+++}	6×10^{-4}	94
Mg^{++}	1×10^{-3}	101
Pb^{++}	6×10^{-4}	86
Cu^{++}	1×10^{-3}	54
	6×10^{-4}	91
	6×10^{-5}	107
Hg^{++}	1×10^{-4}	28
	6×10^{-5}	33
	3×10^{-5}	87
	6×10^{-6}	111

* Each flask contained 2 ml. of lipase emulsion prepared in 0.1 M Tris buffer (pH 7.2), a solution of the metal ion, and water to make a total of 9.5 ml.; this mixture was incubated for 10 minutes; the reaction was initiated by addition of 0.5 ml. of 0.5 M acetate buffer (pH 4.0).

TABLE 2. EFFECT OF ADDITIVES ON ENDOGENOUS HYDROLYSIS BY LIPASE EMULSION *

Additive	Concentration	Length of Incubation	Relative Activity
			per cent
None			100 †
Serum albumin	0.5–2.0%	10 min.	100
Protamine sulfate	150–300 μg./ml.	10 min.	100
Heparin, sodium salt	1 mg./ml.	10 min.	100
N-Ethylmaleimide	0.002 M	15 min.	79
	0.002 M	18 hrs.	53
	0.004 M	18 hrs.	46
p-Chloromercuribenzoic acid	0.001 M	30 min.	0
p-Chloromercuribenzoic acid ‡	0.001 M	30 min.	100
Diisopropylfluorophosphate	0.2 M	18 hrs.	31
	0.025 M	18 hrs.	50
	0.01 M	18 hrs.	106
Cyanide §	0.005 M	10 min.	92
Fluoride §	0.01 M	10 min.	41

* Each flask contained 2 ml. of lipase emulsion prepared in 0.1 M phosphate buffer (pH 7.0), a solution of additive, and water to make a total of 6 ml.; this mixture was incubated for the length of time indicated; the reaction was initiated by addition of 4 ml. of 0.1 M acetate buffer (pH 4.0).

† Appropriate controls with no additives, incubated for the same lengths of time, were used in calculating relative activity.

‡ After 30 minutes of incubation, 0.005 M cysteine was added; 30 minutes later buffer was added to initiate the reaction.

§ Enzyme prepared in Tris buffer.

DISCUSSION

The procedure for isolation of the enzyme, together with its substrate, seems to provide a complete lipase system. This is reflected in the rapidity of the reaction and its completeness without additives. In much of the literature on seed lipases, reactions are carried out for periods of 2 hours to as long as 10 days. It is questionable whether the hydrolytic changes that take place in some of the longer periods are mediated by physiological enzyme systems; more likely these are vestiges of enzyme systems. This system also seems to have a greater stability to water. Moulé (24) has reported that incubation of castor bean pomace in water at 20°C for 1 hour destroyed 90 per cent of its lipase activity; but in the experiments presented here there is no evidence of such extraordinary instability to water, either during storage of the lipase emulsion or during measurement of rate of hydrolysis. Whether the stability of our preparation is due to the presence of substrates or to the method of preparation, which does not involve prior removal of fat, cannot be stated from this work.

Examination of the active emulsions under the microscope and with the use of water- and oil-soluble stains, before and after adjustment to acid pH, indicated that the emulsions are of the oil-in-water type. A freshly prepared lipase emulsion will "cream" after standing for several hours. Nevertheless, this will quickly reconstitute on mixing. When the lipase emulsion has been shaken for several weeks or heated as described previously, it will take on an oily appearance. We have not been able to associate directly this weakening in the emulsifying activity with lowering of lipase activity. The two seem to go hand in hand in experiments on shaking or heating, but it is possible to inhibit the lipase activity entirely by addition of mercuric ion or of PCMB without interfering with its emulsifying properties.

There seems to be every indication that sulfhydryl groups are part of the active system of the enzyme. Although it is not necessary to have cysteine or other reducing agents present for full activity of the enzyme, there is more activity in its presence and with EDTA. It is entirely possible that when the substrate is removed, cysteine or some other reducing agent will be needed to maintain activity, just as similar reducing agents are needed for maintenance of activity of papain (16). The effect of EDTA might be to remove interfering ions, such as copper, which were demonstrated to have an inhibitory effect. The fact that the enzyme is not inhibited by EDTA is also presumptive evidence that no metal coenzymes are part of the system.

The lipase system described herein can obviously serve only as a guidepost for further purification of the enzyme. Preliminary experiments (25) have shown that it is possible to remove the substrate from the lipase emulsion by extraction with ether in the presence of saturated salt solution. The resulting preparation catalyzes hydrolysis of glycerides of long-chain fatty acids without addition of emulsifiers or fatty acid acceptors; here too the course of the reaction can be described by first-order kinetics.

The authors wish to thank Mr. Don S. Bolley, of The Baker Castor Oil Company, for the supply of castor beans.

REFERENCES

1. Altschul, A. M. In *Cottonseed and Cottonseed Products*, edited by A. E. Bailey, New York, Interscience Publishers, Inc., 1948, p. 157.
2. Browne, Jr., C. A. *J. Am. Chem. Soc.* **25**: 948, 1903.
3. Lewkowitsch, J., and G. H. Warburton. *Chemical Technology and Analysis of Oils, Fats and Waxes*, 6th ed., New York, Macmillan, 1922, vol. 2, p. 558.
4. Bamann, E., and E. Ullmann. In *Handbuch der Pflanzenphysiologie*, edited by W. Ruhland, Berlin, Springer-Verlag, 1957, vol. 7, p. 109.
5. Longenecker, H. E., and D. E. Haley. *J. Am. Chem. Soc.* **57**: 2019, 1935.
6. Bolley, D. S., and R. A. Holmes. In *Processed Plant Protein Foodstuffs*, edited by A. M. Altschul, New York, Academic Press, Inc., 1958, p. 840.
7. Rose, W. G. *J. Am. Oil Chemists' Soc.* **28**: 47, 1951.
8. Rodbell, M. *Science* **127**: 701, 1958.
9. Hill, U. T. *Anal. Chem.* **19**: 932, 1947.
10. Bauer, F. C., Jr., and E. F. Hirsch. *Arch. Biochem.* **20**: 242, 1949.
11. Marchis-Mouren, G., L. Sarda and P. Desnuelle. *Arch. Biochem. Biophys.* **83**: 309, 1959.
12. Bamann, E., and E. Ullmann. *Biochem. Z.* **312**: 9, 1942.
13. Haley, D. E., and J. F. Lyman. *J. Am. Chem. Soc.* **43**: 2664, 1921.
14. Guillemet, R. *Compt. rend. soc. biol.* **108**: 781, 1931.
15. Luchsinger, W. W., L. S. Cuendet, P. D. Boyer, and W. D. Geddes. *Cereal Chem.* **32**: 395, 1955.
16. Kimmel, J. R., and E. L. Smith. *J. Biol. Chem.* **207**: 515, 1954.
17. Olcott, H. S., and T. D. Fontaine. *J. Am. Chem. Soc.* **63**: 825, 1941.
18. Sarda, L., and P. Desnuelle. *Biochim. et Biophys. Acta* **30**: 513, 1958.
19. Korn, E. D. *J. Biol. Chem.* **215**: 1, 1955.
20. Korn, E. D., and T. W. Quigley, Jr. *J. Biol. Chem.* **226**: 833, 1957.
21. Rothschild, P. *Biochem. Z.* **206**: 186, 1929.
22. Lipmann, F., and L. C. Tuttle. *Biochim. et Biophys. Acta* **4**: 301, 1950.
23. Desnuelle, P., M. J. Constantin and L. Sarda. *Bull. soc. chim. biol.* **38**: 625, 1956.
24. Moulé, Y. *Oléagineux* **8**: 561, 1953.
25. Altschul, A. M., R. L. Ory and A. J. St. Angelo. *Federation Proc.* **18**: 180, 1959.

J. Lipid Research
April, 1960

Fat metabolism in higher plants: XIII. phosphatidic acid synthesis and diglyceride phosphokinase activity in mitochondria from peanut cotyledons*

CLIVE BRADBEER† and P. K. STUMPF

Department of Biochemistry, University of California, Davis, California

[Received for publication January 8, 1960]

SUMMARY

A phosphatidic acid is the major lipid to become labeled when Pi^{32} (inorganic orthophosphate labeled with P^{32}), under conditions of oxidative phosphorylation, or ATP^{32} is fed to mitocholine and phosphatidylethanolamine in the mitochondria obtained a much higher level of P^{32}, the only cofactor required was Mg^{++}. The stimulation of phosphatidic acid synthesis by an α,β-diglyceride provided support for the view that this synthesis is due to diglyceride phosphokinase activity in the mitochondria. Evidence is also presented that this enzyme preparation is capable of phosphorylating α-monoglycerides with the formation of monoacyl phosphatidic acids. When Pi^{32} was added to slices of peanut cotyledons, the phosphatidylcholine and phosphatidylethanolamine in the mitochondria obtained a much higher level of radioactivity than that observed in experiments with the isolated mitochondria.

Mazelis and Stumpf (1) have shown that fresh mitochondria from the cotyledons of germinating peanut seedlings are capable of incorporating Pi^{32} (inorganic orthophosphate labeled with P^{32}) into a phospholipid fraction. The labeled phospholipid was not identified, but it was established that the first step on the pathway of Pi^{32} incorporation was the esterification of Pi^{32} into ATP^{32}.‡ Fresh, frozen, or lyophilized mitochondria were able to incorporate label from ATP^{32} into the phospholipid fraction and the only cofactor required by these systems was Mg^{++}. Evidence is presented in the present paper to show that the labeled phospholipid is a phosphatidic acid and that the incorporation of label from ATP^{32} into this lipid is due to diglyceride phosphokinase activity in the mitochondria.

* Supported in part by Grant-in-Aid G-4582 from the National Science Foundation.

† Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin.

‡ The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; CMP, cytidine monophosphate; FAD, flavin adenine dinucleotide; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; Co A, coenzyme A; GSH, reduced glutathione; GP, glycerophosphate; GPE, glyceryl phosphoryl ethanolamine; GPC, glyceryl phosphoryl choline; Pi^{32}, inorganic orthophosphate labeled with P^{32} (Pi^{32} solutions were obtained from the Department of Radiation Safety, University of California, Berkeley); ATP^{32}, adenosine triphosphate labeled in the terminal phosphate group with P^{32}.

MATERIAL AND METHODS

Preparation of ATP^{32}. ATP, terminally labeled with P^{32}, was prepared from ADP and Pi^{32} by means of the photophosphorylative activity of isolated spinach chloroplasts. The isolation of the chloroplasts and the reaction conditions used were closely similar to the methods described by Arnon *et al.* (2).[1] Each reaction mixture contained 12 μmoles ADP, 10 μmoles Pi^{32} (1 to 2 mc.), 80 μmoles Tris buffer pH 8.1, 10 μmoles sodium ascorbate, 10 μmoles $MgCl_2$, 0.3 μmoles menadione, and chloroplasts (containing 0.5 to 1 mg. chlorophyll) in a total volume of 3 ml. The reaction mixtures were illuminated for 30 minutes at 15°C. Over 95 per cent of the Pi^{32} was incorporated into ATP^{32} under these conditions. The 10 μmoles of ATP^{32}, containing 1 to 2 mc. of P^{32}, was usually diluted with 50 μmoles of nonradioactive ATP prior to the following isolation steps. The removal of the chloroplasts from the reaction mixture by high speed centrifugation and all the subsequent preparative steps

[1] Acknowledgment is due to Drs. J. Bove and F. R. Whatley for instruction in the preparation of ATP^{32} using spinach chloroplasts.

were carried out at 0°C. The ATP^{32} was precipitated, at neutral pH, as its dibarium salt by the addition of a suitable excess of barium acetate to the chloroplast-free supernatant solution. After two washings with demineralized water, the precipitate was dissolved in the minimum volume of 0.5 N hydrochloric acid and barium ions were removed by the addition of sufficient Dowex 50 (H^+ form). The resin was removed by centrifugation and the ATP^{32} solution was carefully neutralized to pH 7 with M sodium hydroxide. No attempt was made to remove the slight excess of unlabeled ADP or the traces of unchanged Pi^{32} from the preparation. The ATP^{32} was stored at −15°C until required.

Plant Material. Peanuts were germinated in moist vermiculite in the dark at 25°C. Mitochondria were prepared, as described by Stumpf (3), from the cotyledons obtained from 3- to 7-day-old seedlings. The mitochondria, suspended in 0.1 M Tris, 0.5 M sucrose, pH 7.2, were either used immediately or were stored at −15°C until needed.

Reaction Conditions. The mitochondria were usually incubated at 30°C for 60 minutes in a reaction mixture containing 0.1 M Tris, 0.5 M sucrose buffer, of pH 7.2. When ATP^{32} was employed as the source of P^{32}, the only cofactor required was Mg^{++}. In some experiments with freshly prepared mitochondria, Pi^{32} was used and these systems required, in addition, an adenine nucleotide, oxygen, and a tricarboxylic acid cycle intermediate. The precise reaction mixtures used are shown in the appropriate tables. Potassium fluoride, in a final concentration of 0.1 M, was commonly included since it was found to increase the incorporation of label into phospholipid, presumably by means of its inhibition of adenosine triphosphatase.

Lipid Extraction and Analysis. At the end of the reaction period the mitochondria were precipitated by the addition of 2 ml. of 10 per cent trichloroacetic acid. After centrifugation, the precipitate was washed twice with aliquots of 5 per cent trichloroacetic acid and the lipids were then extracted and washed by means of the methods of Kennedy (4), as modified by Mazelis and Stumpf (1).

Aliquots of the lipid extract were counted with a Nuclear-Chicago gas-flow counter fitted with a micromil window.

Portions of the lipid extracts were chromatographed on Whatman 3MM paper impregnated with silicic acid, according to the method of Lea *et al.* (5). The developing solvent was diisobutyl ketone:acetic acid: water (40:30:7, v/v/v) (6).

Deacylation of the phospholipids was carried out by reaction with 0.1 N methanolic potassium hydroxide at 37°C for 15 minutes, according to the method of Dawson (7) as modified by Benson and Maruo (8). After removal of the excess potassium ions by Dowex 50 (H^+ form) and the subsequent removal of the resin, the phosphate esters were chromatographed in two dimensions on acid-washed Whatman No. 1 paper. The developing solvents were water-saturated phenol and *n*-butanol : propionic acid : water (142 : 71 : 100, v/v/v). In addition, methyl cellosolve : methyl ethyl ketone : 3 N ammonium hydroxide (7 : 2 : 3, v/v/v) and isobutanol : picric acid : water (80 ml. : 2 g. : 20 ml.) were occasionally used for one-dimensional chromatography.

Phosphates were detected on paper by the modification of the Hanes and Isherwood procedure (9) described by Bandurski and Axelrod (10). Lipids were demonstrated with 0.001 per cent aqueous Rhodamine B (11) and compounds with an amino group were located with 0.2 per cent ninhydrin in acetone.

Radioactive compounds were detected on paper by radioautography with Kodak "No Screen" X-ray film.

RESULTS

In some preliminary experiments the results of Mazelis and Stumpf (1), concerning the necessary conditions for the incorporation of Pi^{32} and ATP^{32} into phospholipid by peanut mitochondria, were confirmed. The stimulatory effect of Mg^{++} upon the incorporation of label from ATP^{32} into phospholipid by frozen peanut mitochondria was again demonstrated. There was some residual activity in the absence of added Mg^{++} and this was probably due to Mg^{++} contained in the mitochondria. Mazelis and Stumpf (1) showed that this residual activity could be abolished by the addition of versene to the enzyme system and that activity could be restored by the subsequent addition of either Mg^{++} or Mn^{++}. The effect of high concentrations of Mg^{++} in the reaction mixture was not investigated. Mg^{++} was the only cofactor required for the incorporation of label from ATP^{32} into the phospholipid by fresh or frozen mitochondria. The incorporation of Pi^{32}, on the other hand, only occurred under conditions favorable for oxidative phosphorylation, i.e., with fresh mitochondria, Mg^{++}, an adenine nucleotide, a tricarboxylic acid cycle intermediate, and oxygen.

Identification of the Labeled Phospholipid. The main aim of the present study was to identify the phospholipid(s) which became labeled in peanut mitochondria under the experimental conditions described by Mazelis and Stumpf (1).

When portions of the lipid extract were chromatographed on silicic acid-impregnated paper with diisobutyl ketone-acetic acid-water as the developing solvent, approximately 80 to 90 per cent of the radioactivity ran with an R_f value of 0.78. This mobility most closely resembles that of a diacyl phosphatidic acid, for which R_f values of 0.72 (6) and 0.84 (12) have been reported. The remaining 10 to 20 per cent of the radioactivity had an R_f of 0.52.

Aliquots of the lipid extract were deacylated by the method described and the residual phosphate esters were chromatographed both in one and in two dimensions on acid-washed Whatman No. 1 paper. Between 96 and 99 per cent of the radioactivity was located in a single spot, which was identified as α-glyerophosphate by cochromatography with authentic L,α-glycerophosphate[2] in the four solvents used. This compound was finally proved to be α-glycerophosphate by periodate oxidation according to the method of Baddiley *et al.* (13). Chromatography of the oxidation products on Whatman No. 1 paper, with isobutanol : picric acid : water as the developing solvent, showed that the radioactivity had entirely disappeared from the α-glycerophosphate position (R_f 0.23) and now cochromatographed precisely with the glycolaldehyde phosphate (R_f 0.51), which was formed by the simultaneous periodate oxidation of the standard α-glycerophosphate. This identification of α-glycerophosphate as the product of deacylation identifies the original radioactive lipid as being a phosphatidic acid. This finding is consistent with the observed mobility of the lipid on silicic acid-impregnated paper, as described earlier.

It should be noted that the methods used would clearly differentiate a phosphatidic acid from bisphosphatidic acids and from phosphatidyl glycerols. Deacylation of the two latter groups of compounds would yield, in both cases, glycerol phosphoryl glycerol, which would be readily separated from α-glycerophosphate under the paper chromatographic conditions employed.

The fact that over 96 per cent of the label of the deacylated phosphate esters was in α-glycerophosphate indicates that the lipid, which contained 10 to 20 per cent of the radioactivity in the original lipid extract and which chromatographed with an R_f of 0.52 in the silicic acid system, must also give rise to some α-glycerophosphate upon deacylation. These facts have been taken to indicate that part of the radioactivity at this position on the silicic acid-impregnated chromatogram is due to a monoacyl phosphatidic acid. Further evidence in support of this suggestion is presented below.

Small amounts of radioactivity (less than 4 per cent) in the deacylated extract were found in a compound which was identified as glyceryl phosphoryl ethanolamine by cochromatography with the authentic substance. From the known mobility of the parent phosphatidylethanolamine, from which this phosphate ester is derived, it has been concluded that labeled phosphatidylethanolamine contributes some radioactivity to the radioactive lipid spot which has an R_f of 0.52 on the silicic acid-impregnated paper. This lipid spot would then consist of a mixture of a monoacyl phosphatidic acid and phosphatidylethanolamine. Attempts were made to stimulate the incorporation of label from ATP^{32} into phosphatidylethanolamine by the inclusion of CTP, CMP, α,β-diglyceride, ethanolamine, and a supernatant protein fraction (obtained by 70 per cent ammonium sulfate saturation of the supernatant solution which remained after removal of the mitochondria from the peanut cotyledon homogenate) in the reaction mixtures. The incorporation of the radiophosphorus into the phosphatidylethanolamine remained low in all cases, as shown in Table 1.

Traces of radioactivity were occasionally observed in other areas on the two-dimensional chromatograms of the deacylated lipid extract, but these spots did not correspond in position to any well-known phosphate esters, and were not identified.

The evidence indicates, however, that when ATP^{32} is fed to mitochondria from peanut cotyledons, the main lipid which becomes labeled is a phosphatidic acid. There is also a small amount of radioactivity in phosphatidylethanolamine.

Diglyceride and Monoglyceride Phosphokinase Activity. Having identified the labeled phospholipid as being predominantly a phosphatidic acid, the next step was to identify the step or steps by which P^{32} is incorporated into this compound. The first possibility to be considered was that the phosphatidic acid had arisen via an initial phosphorylation and subsequent acylation of glycerol, as has been described by Kornberg and Pricer (14) and by Kennedy (15). However, from isotope dilution experiments with unlabeled glycerophosphate and from experiments with C^{14}-labeled glycerol, Mazelis and Stumpf (1) showed that glycerophosphate was not an intermediate in the formation of their labeled phospholipid. The ob-

[2] Thanks are due to Dr. C. E. Ballou for samples of α- and β-glycerophosphate; to Dr. E. Baer for samples of dipalmitoyl L,α-glycerophosphoric acid and L,α-glyceryl phosphoryl ethanolamine; to Dr. D. J. Hanahan for samples of glyceryl phosphoryl choline, α,β-diglyceride (C_{16} unsaturated) and phosphatidylinositol; and to Dr. J. G. Marr for a sample of α-mono palmitin.

TABLE 1. ATTEMPTS TO INCREASE P^{32} INCORPORATION INTO CEPHALIN BY PEANUT MITOCHONDRIA *

	Source of P^{32}			
	Pi^{32}		ATP^{32}	
Tube Number	1	2	3	4
cpm. in lipid fraction	63,618	69,184	72,811	91,114
Proportions of P^{32} in GP and GPE after deacylation of lipid extract: GP	100	100	100	100
GPE	4	4	10	1

* Tubes 1 and 2 contained 10 ml. fresh mitochondria, $MgCl_2$ (10 μmoles), GSH (10 μmoles), 1 mg. each of FAD, DPN, TPN, ATP, Co A, cytochrome c, ADP (10 μmoles), succinate (20 μmoles), Pi^{32} (4 μmoles), and KF (200 μmoles). In addition, tube 2 also contained dipalmitolein (5 μmoles), ethanolamine (10 μmoles), serine (10 μmoles), choline (10 μmoles), CTP (0.5 μmoles), CMP (0.5 μmoles) in a total volume of 4.3 ml. Tubes 3 and 4 contained 1 ml. fresh mitochondria, $MgCl_2$ (10 μmoles), GSH (10 μmoles), ATP^{32} (4 μmoles), KF (200 μmoles), dipalmitolein (5 μmoles), ethanolamine (10 μmoles), serine (10 μmoles), choline (10 μmoles), CTP (0.5 μmoles), CMP (0.5 μmoles) in a total volume of 3.7 ml. Tube 4 also contained 1.5 ml. of the supernatant solution which remained after removal of the mitochondria from the homogenized cotyledons. All tubes incubated at 30°C for 60 minutes.

jection that the glycerophosphate may not have penetrated the mitochondria can be ruled out since Stumpf (3) has shown that glycerophosphate is readily metabolized by these particles. A second possibility is that the phosphatidic acid may have arisen by phosphorylation of a diglyceride by means of a diglyceride phosphokinase such as that which has been demonstarted in particles from guinea pig brain tissue by Hokin and Hokin (16).

The addition of an α,β-diglyceride to the reaction mixtures markedly stimulated the incorporation of label from ATP^{32} into the lipid fraction (Tables 2 and 3). Chromatography of the lipid extract on silicic acid-impregnated paper showed that the distribution of the radioactivity was essentially the same as that obtained in the experiments without added diglyceride, i.e., the bulk of the activity ran to the same place as the component, which has been identified as a diacyl phosphatidic acid, and the remainder chromatographed with the fraction which is believed to consist of a mixture of monoacyl phosphatidic acid and phosphatidylethanolamine. Deacylation of the lipids showed that, as before, almost all of the radioactivity was contained in α-glycerophosphate, thus giving a clear demonstration of the fact that the diglyceride stimulated incorporation of label from ATP^{32} into phosphatidic acid.

Strong evidence that the radioactive lipid of R_f 0.52 on the silicic acid-impregnated paper is mainly a monoacyl phosphatidic acid was obtained when α-

TABLE 2. THE EFFECTS OF DI- AND TRI-GLYCERIDE UPON THE INCORPORATION OF P^{32} FROM ATP^{32} INTO LIPID BY PEANUT MITOCHONDRIA *

Tube Number	Additions		Lipid Extract
		μmoles	*cpm.*
1		0	43,740
2	α, β-dipalmitolein	7	82,660
3		0	22,722
4	α, β-dipalmitolein	5	40,695
5	Triolein	5	36,057
6	Triolein	5	31,027

* Tubes 1 and 2 contained 1 ml. mitochondrial preparation, $MgCl_2$ (2 μmoles), and ATP^{32} (8 μmoles, 0.1 mc.) in a total volume of 1.8 ml. Tubes 3, 4, 5, and 6 contained 0.5 ml. mitochondrial preparation, ATP^{32} (4 μmoles, 40 μc.), $MgCl_2$ (10 μmoles), KF (150 μmoles), and GSH (10 μmoles) in a total volume of 1.6 ml. All tubes incubated at 30°C for 60 minutes.

TABLE 3. EFFECTS OF MONO-, DI-, AND TRI-GLYCERIDES AND OF PHOSPHATIDIC ACID ON THE INCORPORATION OF P^{32} FROM ATP^{32} INTO LIPID BY PEANUT MITOCHONDRIA *

Additions	Lipid Extract	Per Cent of P^{32} in Lipid Fraction on Silicic Acid-impregnated Paper with R_f of:	
		0.78	0.52
	cpm.		
	36,419	87.1	12.9
α, β-dipalmitolein	77,046	86.4	13.6
Dipalmitoyl phosphatidic acid	21,630	58.4	41.6
α-monopalmitin	28,731	17.1	82.9
Triolein	99,416	82.4	17.6

* Each reaction mixture contained 1 ml. mitochondrial preparation, ATP^{32} (3 μmoles), $MgCl_2$ (10 μmoles), and KF (200 μmoles) in a total volume of 2.3 ml. Ten μmoles of emulsified lipids was added appropriately as indicated above. All reaction mixtures incubated at 30°C for 60 minutes.

monopalmitin was included in the reaction mixture. Although the monopalmitin caused some inhibition of the over-all P^{32} incorporation into the lipid fraction, over 80 per cent of the incorporated label was now found at the position of the suspected monoacyl phosphatidic acid (i.e., at R_f 0.52) rather than at the position of the diacyl phosphatidic acid (R_f 0.78) (Table 3). Since deacylation of this lipid extract gave α-glycerophosphate as essentially the only radioactive compound, this effect of the monopalmitin can be explained only in terms of monoglyceride phosphokinase activity to give a monoacyl phosphatidic acid.

As shown in Tables 2 and 3, the incorporation of P^{32} from ATP^{32} into phosphatidic acid was also markedly stimulated by triolein and the proportions of label in the diacyl and monoacyl compounds were approximately the same as those obtained when diglyceride was added. It is suggested that the triglyceride is broken down to a diglyceride which is subsequently phosphorylated, although the triolein was not checked for the initial presence of a diglyceride.

Dipalmitoyl phosphatidic acid was found to inhibit the incorporation of label from ATP^{32} into the lipid fraction (Table 3).

Acetone Powders. Acetone powders of the peanut mitochondria were prepared by the method described by Martin and Stumpf (17). Five per cent suspensions of these powders in 0.2 M Tris buffer, pH 7.2, were effective in incorporating label from ATP^{32} into phosphatidic acid, which was identified by the methods described previously. Table 4 shows the stimulation, by diglyceride, of the incorporation of the label into the lipid fraction. Removal of the insoluble matter from the acetone powder suspensions by centrifugation gave water-clear extracts which still possessed some diglyceride phosphokinase activity, although this activity was much less than that of the suspensions. The stimulation of this system by diglyceride is also shown in Table 4.

Slices of Peanut Cotyledons. Experiments were performed in which Pi^{32} was added to slices of cotyledons obtained from 5- to 6-day-old peanut seedings. Usually about 10 g. fresh weight of plant material was used and the slices were immersed in approximately 12 ml. of 0.1 M Tris, 0.5 M sucrose buffer, pH 7.2 contained in 50 ml. Erlenmeyer flasks. To these systems was added 1 to 2 mc. of Pi^{32} (pH 7.2, 15 to 30 μmoles). The slices were then incubated at 30°C for periods which varied from 6 to 18 hours. At the end of these periods the slices were washed, mixed with 30 to 40 g. of fresh peanut cotyledons, and the mitochondria were separated as described previously. The lipids were extracted from the mitochondria and were subjected to the same analytical procedures as before. Chromatography of the phosphate esters obtained after deacylation of the lipid extract showed five major radioactive areas. The R_f values of these spots in the phenol and butanol-propionic solvents and the proportions of radioactivity which they contained, after a typical experiment, are shown in Table 5. Three of these compounds have been identified as α-glycerophosphate, glyceryl phosphoryl ethanolamine, and glyceryl phosphoryl choline by cochromatography with the authentic substances. Both the unknown esters and the standard compounds chromatographed as discrete spots in this two-dimensional, paper chromatographic system. The unknown I has been tentatively identified as glyceryl phosphoryl inositol because it cochromatographed with the phosphate ester formed by deacylation of a phosphatidyl inositol. The unknown II was not identified but its chromatographic properties are similar to those of glyceryl phosphoryl glycerol as described by Benson and Maruo (8).

DISCUSSION

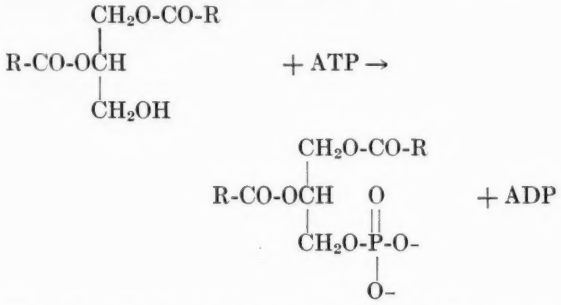
The unknown phospholipid, described by Mazelis and Stumpf (1), and which became labeled when peanut mitochondria were exposed to Pi^{32} or to ATP^{32} under suitable conditions, has been identified as a phosphatidic acid. The results are similar to those which have been described by Hokin and Hokin (18),

TABLE 4. EFFECTS OF DI- AND TRI-GLYCERIDES ON THE INCORPORATION OF P^{32} FROM ATP^{32} INTO LIPID BY ACETONE POWDERS OF PEANUT MITOCHONDRIA *

Acetone Powder					
Suspended			Water-clear Extract		
Additions	*μmoles*	*cpm.*	*Additions*	*μmoles*	*cpm.*
	0	4080		0	294
α, β-dipalmitolein	5	9888	α, β-dipalmitolein	5	1264
Triolein	5	4740			

* With the acetone powder suspension, each reaction mixture contained 0.5 ml. enzyme preparation (50 mg. acetone powder suspended/ml. 0.2 M Tris buffer, pH 7.2), ATP^{32} (40 μmoles, 40 μc.), $MgCl_2$ (10 μmoles), KF (100 μmoles) in a total volume of 1.3 ml. With water-clear extract, each reaction mixture contained 0.35 ml. enzyme preparation (50 mg. acetone powder suspended/ml. 0.2 M Tris buffer, pH 7.2, and insoluble material removed by centrifugation), ATP^{32} (4 μmoles, 40 μc.), $MgCl_2$ (10 μmoles), and KF (100 μmoles) in a total volume of 1 ml. All reaction mixtures incubated at 30°C for 60 minutes.

in whose experiments, under conditions of oxidative phosphorylation, Pi^{32} was readily incorporated into phosphatidic acid by a particulate fraction from guinea pig brain hemispheres. The work of Mazelis and Stumpf (1) has clearly demonstrated that glycerophosphate is not an intermediate in this formation of phosphatidic acid by peanut mitochondria. The stimulation of the phosphatidic acid synthesis by diglyceride has provided evidence that this process takes place by direct phosphorylation of an α,β-diglyceride, as shown in the following reaction:



The only cofactor requirement is for Mg^{++}. The enzyme that catalyzes this reaction has been given the name diglyceride phosphokinase by Hokin and Hokin (16), who showed that such activity was contained in deoxycholate extracts of microsomes obtained from guinea pig brain tissue. The peanut enzyme system is also capable of phosphorylating α-monoglycerides with the formation of monoacyl phosphatidic acids.

Further work is required to determine the relationships of phosphatidic acid synthesis in peanut mitochondria to the metabolism of other phospholipids and to the function of the mitochondrion as a whole. From their work with guinea pig brain tissue, Hokin and Hokin (19) have obtained evidence that phosphatidic acid can function as a carrier for sodium ions across intracellular membranes. The similarity of behavior between the peanut mitochondria and the guinea pig particles of Hokin and Hokin, with regard to the formation of phosphatidic acid by means of a diglyceride phosphokinase, might suggest a similar close relationship in the function of the phosphatidic acid.

The lipid extracts from the peanut mitochondrial experiments usually contained small amounts of radioactivity in phosphatidylethanolamine. However, a much higher proportion of label in phosphatidylethanolamine was obtained in the mitochondria when Pi^{32} was added to slices of the peanut cotyledons (Table 5). Kennedy (15) has summarized the evidence that phosphatidylethanolamine is synthesized in animal tissues by means of the steps centered below:

Ethanolamine + ATP → phosphoethanolamine + ADP
Phosphoethanolamine + CTP → CMP-phosphoethanolamine + pyrophosphate
CMP-phosphoethanolamine + α,β-diglyceride → phosphatidylethanolamine + CMP

TABLE 5. RADIOACTIVE PHOSPHATE ESTERS OBTAINED BY DEACYLATION OF THE LIPID EXTRACT FROM MITOCHONDRIA SEPARATED FROM SLICES OF PEANUT COTYLEDONS EXPOSED TO Pi^{32} *

Phosphate Ester	R_f in Butanol/Propionic Acid/Water (40/30/7)	R_f in Phenol	
			cpm.
GP	0.17	0.26	4421
GPE	0.19	0.57	3526
GPC	0.23	0.83	693
I	0.08	0.10	2898
II	0.13	0.38	1684

* Ten g. fresh weight of peanut cotyledon slices incubated in 12 ml. 0.1 M Tris, 0.5 M sucrose, pH 7.2, containing Pi^{32} (1.7 mc., 28 μmoles) at 30°C for 18 hours. Lipids extracted from the mitochondria and then deacylated and residual phosphate esters chromatographed on acid-washed Whatman No. 1 paper. The cpm. given are those obtained from a typical two-dimensional chromatogram of an aliquot of the phosphate esters.

Accordingly, with isolated peanut mitochondria, attempts were made to increase the porportion of label in phosphatidylethanolamine by the incorporation of ethanolamine, CTP, and diglyceride into the reaction mixtures. These attempts were not successful. This may be due to loss of activity during the preparation of the mitochondria, or to the fact that phosphatide synthesis in plants follows a different pathway from that in animal tissues.

REFERENCES

1. Mazelis, M., and P. K. Stumpf. *Plant Physiol.* **30**: 237, 1955.
2. Arnon, D. I., M. B. Allen and F. R. Whatley. *Biochim. et Biophys. Acta* **20**: 449, 1956.
3. Stumpf, P. K. *Plant Physiol.* **30**: 55, 1955.
4. Kennedy, E. P. *J. Biol. Chem.* **201**: 399, 1953.
5. Lea, C. H., D. N. Rhodes and R. D. Stoll. *Biochem. J.* **60**: 353, 1955.

6. Marinetti, G. V., and E. Stotz. *Biochim. et Biophys. Acta* **21**: 168, 1956.
7. Dawson, R. M. C. *Biochim. et Biophys. Acta* **14**: 374, 1954.
8. Benson, A. A., and B. Maruo. *Biochim. et Biophys. Acta* **27**: 189, 1958.
9. Hanes, C. S., and F. A. Isherwood. *Nature* **164**: 1107, 1949.
10. Bandurski, R. S., and B. Axelrod. J. *Biol. Chem.* **193**: 405, 1951.
11. Rouser, G., G. V. Marinetti, R. F. Witter, J. F. Berry, and E. Stotz. *J. Biol. Chem.* **223**: 485, 1956.
12. Hokin, L. E., and M. R. Hokin. *J. Biol. Chem.* **233**: 805, 1958.
13. Baddiley, J., J. G. Buchanan, A. P. Mathias, and A. R. Sanderson. *J. Chem. Soc.*, 4186, 1956.
14. Kornberg, A., and W. E. Pricer, Jr. *J. Biol. Chem.* **204**: 329, 345, 1953.
15. Kennedy, E. P. *Ann. Rev. Biochem.* **26**: 119, 1957.
16. Hokin, L. E., and M. R. Hokin. *Biochim. et Biophys. Acta* **31**: 285, 1959.
17. Martin, R. O., and P. K. Stumpf. *J. Biol. Chem.* **234**: 2548, 1959.
18. Hokin, L. E., and M. R. Hokin. *J. Biol. Chem.* **233**: 822, 1958.
19. Hokin, L. E., and M. R. Hokin. *Nature* **184**: 1068, 1959 (Suppl.).

Volume 1
Number 3

Studies on the distribution of tocopherol in human serum lipoproteins*

EUGENE C. MCCORMICK,† DAVID G. CORNWELL, and J. B. BROWN

Department of Physiological Chemistry,
The Ohio State University, Columbus 10, Ohio

[Received for publication August 26, 1959]

SUMMARY

The tocopherol content of human serum proteins, isolated by density gradient and homogeneous density procedures, was determined by the Emmerie-Engel and phosphomolybdic acid methods. Serum tocopherol is found primarily in the lipoproteins. The low density lipoproteins, principally the S_f 3-9 lipoproteins, contain a major part of the total serum tocopherol, while high density lipoproteins contain the remainder. Serum tocopherol increases after oral administration and remains elevated for 24 hours. The increment in serum tocopherol from newly absorbed tocopherol is found first in the chylomicron and S_f 10-400 lipoprotein fractions. A tocopherol peak occurs in these fractions 4 hours after oral administration. This peak is followed by an increment in the tocopherol content of the S_f 3-9 and high density lipoprotein fractions which reaches a maximum plateau in from 8 to 10 hours and remains elevated from 14 to 16 hours. The distribution of newly absorbed tocopherol is discussed in relation to lipid absorption and transport of other lipids by the serum lipoproteins.

The distribution of tocopherol in the human serum lipoproteins has received limited study. There appears to be a controversy as to whether tocopherol is bound to all the serum proteins (1), bound to low density lipoproteins (2), or bound principally to high density lipoproteins (3). In the present study the distribution of tocopherol in the serum proteins was observed before, during, and after the oral administration of tocopherol to humans.

METHODS

Separation of Lipoproteins. Lipoprotein fractions were separated from human serum by the ultracentrifugal flotation procedure of Havel *et al.* (4). Serum was adjusted to a density of 1.21 g. per ml. with a sodium chloride-potassium bromide stock solution and centrifuged from 18 to 22 hours at 100,000 $\times$ g in a Spinco Model L ultracentrifuge. The low and high density lipoproteins floated to the top of the centrifuge tube and were then separated from the other serum proteins in the infranatant solution with a tube cutter. In the separation of low and high density lipoproteins, serum was adjusted to a density of 1.063 g. per ml. with a sodium chloride-potassium bromide stock solution and centrifuged from 18 to 22 hours at 100,000 $\times$ g.

Low density lipoproteins floated to the top of the centrifuge tube, while high density lipoproteins and other serum proteins remained in the infranatant solution.

Chylomicrons, S_f 10-400, S_f 3-9, and high density lipoproteins were separated by a density gradient ultracentrifugation procedure (5, 6). Five ml. of serum was placed in a 13.5 ml. lusteroid centrifuge tube, overlaid with 4 ml. of 0.15 M sodium chloride,[1] and centrifuged for 30 minutes at 9,300 $\times$ g in the Spinco Model L ultracentrifuge. Chylomicrons formed a turbid band at the top of the saline layer. This band was separated from the infranatant serum with a tube cutter. The infranatant serum was then layered over 5.5 ml. of 2.0 M sodium chloride in a second centrifuge tube, overlaid with 0.15 M sodium chloride to fill the tube, and centrifuged at 100,000 $\times$ g for 18 to 22 hours. The S_f 10-400 lipoproteins were concentrated in a turbid band at the top of the tube, the S_f 3-9 lipoproteins formed a clear orange-yellow band in the center, while high density lipoproteins and other serum proteins sedimented to the bottom of the tube.

* Supported in part by Research Grant H-2807 from the National Institutes of Health.

† Fellow of the Central Ohio Heart Association.

[1] All salt solutions added in the preparation of lipoproteins contained 0.05 g./l. of the disodium salt of ethylenediaminetetraacetic acid adjusted to pH 7.0 $\pm$ 0.2 with 1 N sodium hydroxide (5).

Tocopherol Administration. Two g. of tocopherol[2] was given to young healthy adults with a lipid meal consisting of one-half pint of ice cream and 4 ounces of eggnog. The lipid meal produced an alimentary lipemia. Control experiments were run with the lipid meal alone and with the tocopherol alone. When given without a lipid meal, the dose of tocopherol was reduced to 600 mg. in order to prevent diarrhea. A blood specimen was drawn before and at 2- to 4-hour intervals after the lipid meal. Blood specimens were obtained over a 24-to-48-hour period. Four ml. of serum was used for total serum tocopherol analysis and 5 to 6 ml. for the separation of lipoprotein fractions. Two subjects designated as I and II were used throughout the experimental series for the study of tocopherol absorption and distribution in lipoproteins. Subject I was a 28-year-old healthy male and subject II was a 24-year-old healthy female.

Tocopherol Assay. For the determination of serum tocopherol and its distribution in the serum lipoprotein fractions, the phosphomolybdic acid and Emmerie-Engel (EE) reactions were compared. A modification of the Rosenkrantz procedure (7) was used for the phosphomolybdic acid reaction (PMA). Four ml. of serum or a lipoprotein fraction isolated from 4 ml. of serum was denatured with an equal volume of ethanol and extracted with 15 ml. of isooctane. A 10 ml. aliquot of the isooctane solution was placed in a 50 ml. boiling flask and dried under nitrogen at 40°C in a rotary vacuum evaporator. The residue was first dissolved in 2 ml. of repurified butanol and then 2 ml. of fresh 1.6 per cent phosphomolybdic acid in glacial acetic acid was added. This sample was transferred to a 15 × 150 mm. Pyrex test tube, placed in a 55°C water bath for 15 minutes, and then cooled at room temperature for 30 minutes. Absorbance was determined at 725 mμ. with a Beckman B spectrophotometer. Since carotenoids interfere in the PMA reaction, the carotenoid content of the isooctane extract was estimated from the absorbance of an isooctane aliquot at 450 mμ. Standards of α-tocopherol, the major tocopherol constituent of serum (8), and β-carotene were used in each set of determinations.

Phospholipids and cholesterol interfere in the PMA reaction. Lecithin, isolated from egg yolk by silicic acid chromatography, produces a white precipitate with phosphomolybdic acid upon cooling. Turbid samples were filtered through a pressure filter system consisting of a 10 ml. glass syringe containing two layers of Whatman No. 1 and two layers of Whatman No. 42 filter paper. Phosphomolybdic acid reacts with free cholesterol at incubation temperatures above 60°C and enhances the absorbance at 725 mμ. The free cholesterol in serum inhibits the PMA-tocopherol color reaction by 4 to 5 per cent at incubation temperatures of 55°C. This cholesterol inhibition of the PMA reaction was corrected by the use of tocopherol internal standards. Cholesterol palmitate (recrystallized: m.p. 77°C) had no effect on the PMA reaction.

The PMA reaction measures total tocopherol. When α-tocopherol quinone, prepared by the silver nitrate oxidation procedure of Cuthbertson *et al.* (9), was assayed with the modified PMA procedure, 69 per cent of the color expected from α-tocopherol was obtained. This corresponds to the value obtained for α-tocopherol quinone in the unmodified Rosenkrantz procedure (7).

The ferric chloride-α,α-bipyridine reaction as modified by Farber *et al.* (10) was used for the EE reaction. Four ml. of serum or a lipoprotein fraction was denatured with an equal volume of ethanol and extracted with 4 ml. of xylene for 10 minutes in a mechanical shaker. The emulsion was broken by centrifugation and 3 ml. of the xylene extract transferred to a 19 mm. Coleman cuvette. Three ml. of 0.1 per cent α,α-bipyridine in *n*-propanol was added and the carotenoid absorbance determined at 440 mμ. with a Coleman Nephelo-Colorimeter. Then 0.5 ml. of 0.1 per cent ferric chloride was added, the sample shaken, and read at 525 mμ. after a 2-minute interval. The color developed with pure α-tocopherol was stable from 30 seconds to 10 minutes; however, the color reaction with β-carotene increased with time.

Chemical Analysis. Lipoprotein fractions were placed in volumetric flasks and the lipid extracted with alcohol-ether (3:1). Cholesterol was determined by the method of Abell *et al.* (11). Lipid phosphorus was determined by the method of Lowry *et al.* (12) and multiplied by 25 to estimate phospholipid. Fatty acid ester equivalents were determined by the method of Stern and Shapiro (13).

RESULTS

The Distribution of Tocopherol in Serum Proteins. The fasting serum tocopherol levels are higher when measured by the PMA than by the EE assay method (Tables 1 and 2). Since the PMA reaction estimates tocopherol quinone as well as free tocopherol, these values are higher and are consistent with the report (14) that approximately 30 per cent of serum tocopherol is in the quinone form. No significant difference

[2] Eprolin®, lipid soluble tocopherol, Eli Lilly Co., Indianapolis, Ind.

TABLE 1. TOCOPHEROL DISTRIBUTION IN LOW AND HIGH DENSITY LIPOPROTEIN FRACTIONS OBTAINED BY ULTRACENTRIFUGAL FLOTATION *

Fraction	A	B	C
	Tocopherol in μg./ml. and per cent (*italics*) of total recovered		
Serum	13.8	9.2	23.3
Low and high density lipoproteins (D < 1.21)	13.3 *83*	5.8 *87*	21.7 *94*
Other serum proteins	2.7 *17*	0.9 *13*	1.4 *6*
Total recovered	16.0	6.7	23.1
Percentage recovered	*116*	*72*	*99*
Low density (D < 1.063) lipoproteins	9.6 *66*	5.9 *63*	16.8 *71*
High density lipoproteins and other serum proteins	5.0 *34*	3.5 *37*	7.4 *29*
Total recovered	14.6	9.4	24.2
Percentage recovered	*106*	*102*	*104*

* Ultracentrifugal flotation procedure of Havel *et al.* (4).
A. Subject I, fasting serum, PMA analysis.
B. Subject II, fasting serum, EE analysis.
C. Subject II, 10 hours after test meal with tocopherol, EE analysis.

was found between the tocopherol content of serum and plasma by either assay method.

Serum was fractionated into low and high density lipoproteins by the homogeneous density method and these fractions were analyzed for their tocopherol content by the PMA and EE reactions (Table 1). Over 83 per cent of the tocopherol was found in the serum lipoprotein fractions. The relative amount of tocopherol associated with other serum proteins decreased from 13 per cent to 6 per cent after the oral administration of tocopherol (Table 1). From 63 to 71 per cent of the serum tocopherol was carried by the chylomicrons and low density lipoproteins.

Serum was fractionated into chylomicrons, S_f 10-400, S_f 3-9, and high density lipoproteins by the density gradient method and these fractions were analyzed for their tocopherol content by the PMA and EE reactions (Table 2). The chylomicrons and low density lipoproteins, isolated by the density gradient procedure, contained from 65 to 72 per cent of the serum tocopherol. This tocopherol distribution corresponds to the tocopherol distribution obtained in the separation of low density lipoproteins by the homogeneous density centrifugation procedure (Table 1). The S_f 3-9 lipoprotein fraction contained from 41 to 58 per cent of the serum tocopherol. Tocopherol activities were also determined on lipid extracts from the lipoproteins of subject I by the carotenoid stability test (15). This procedure showed that 69 per cent of the total tocopherol activity was in the S_f 3-9 lipoprotein fraction. Since the PMA and EE reactions showed a similar tocopherol distribution, no difference was detected between the relative content of tocopherol and tocopherol quinone in the serum lipoprotein fractions.

Tocopherol analyses by the PMA reaction on duplicate lipoprotein fractions varied ± 0.1 μg. per ml. to ± 0.4 μg. per ml. from the average of the two determinations. Tocopherol analyses by the EE reaction on duplicate lipoprotein fractions varied ± 0.2 μg. per ml. to ± 0.4 μg. per ml. from the average of the two

TABLE 2. Tocopherol Distribution in Chylomicron, S_f 10-400, S_f 3-9, and High Density Lipoprotein Fractions Obtained by Ultracentrifugal Flotation *

Fraction	A	B	C	D	E
	Tocopherol in μg./ml. and per cent (*italics*) of total recovered				
Serum	12.2	9.1	7.35	6.34	13.1 ± 2.7
Chylomicron	0.14 *1.3*	0.47 *5.2*	0 *0*	0.12 *1.8*	0.51 ± 0.34 *4.0 ± 2.7*
S_f 10-400	1.09 *11*	1.67 *18*	0.67 *9.3*	0.61 *9.8*	1.97 ± 0.62 *15.5 ± 4.9*
S_f 3-9	5.34 *53*	4.38 *48*	4.18 *58*	3.53 *57*	5.96 ± 0.75 *47.0 ± 5.9*
High density lipoproteins and other serum proteins	3.54 *35*	2.58 *28*	2.38 *33*	1.95 *32*	4.24 ± 0.48 *33.4 ± 3.8*
Total recovered	10.1	9.1	7.23	6.21	12.7 ± 1.2
Percentage recovered	*83*	*100*	*98*	*98*	*97 ± 8.7*

* Density gradient ultracentrifugal flotation procedure (5, 6).
A. Subject I, fasting serum, PMA analysis.
B. Subject I, fasting serum, EE analysis.
C. Subject II, fasting serum, PMA analysis.
D. Subject II, fasting serum, EE analysis.
E. 10 subjects, nonfasting serum, PMA analysis, mean ± standard deviation.

determinations. The range in tocopherol content represents the error from both the tocopherol assay and the lipoprotein fractionation.

Tocopherol Distribution During the Active Absorption of Tocopherol. The concentration of serum tocopherol after oral tocopherol administration to subjects I and II is shown in Figure 1. When 2.0 g. of tocopherol was administered to subject II with a lipid meal, the increment in serum tocopherol reached one peak in 6 hours and a second peak in 12 hours. The second peak occurred 3.5 hours after the subject had eaten a regular meal and may represent absorption of unabsorbed tocopherol or reabsorption of biliary excreted tocopherol (16). When 600 mg. of tocopherol was administered to subject I without a lipid meal, a smaller but significant increment in serum tocopherol was obtained. Tocopherol was administered without a lipid meal in order to demonstrate an elevation in serum tocopherol without the parallel elevation of serum lipids, which was verified by phospholipid analysis of the lipoprotein fractions. Serum tocopherol remained elevated for the duration of the test period in both experiments in which supplementary tocopherol was given. When a lipid meal without supplementary tocopherol was given to subject I, no rise in serum tocopherol occurred (Fig. 1).

Serum lipoprotein fractions were separated by the density gradient ultracentrifugation procedure, and tocopherol increments in the lipoprotein fractions were measured at stated time intervals for the duration of the test period. The chylomicron and S_f 10-400 lipoprotein fractions showed a peak tocopherol increment 4 hours after tocopherol administration (Figs. 2 and 3). When 2.0 g. of tocopherol was administered to the subject, a second peak occurred in the chylomicron and S_f 10-400 lipoprotein fractions 3 to 4 hours after the subject had eaten a second meal (Fig. 2). A single peak in the tocopherol increment was found for both the chylomicron and S_f 10-400 lipoprotein fractions when 600 mg. of tocopherol was administered to a subject (Fig. 3). The tocopherol content of the S_f 3-9 lipoprotein fraction reached a maximum from 6 to 10 hours after the test meal and maintained a plateau for another 12 hours (Figs. 2 and 3). The tocopherol increment in the high density lipoprotein fraction follows a pattern similar to that in the S_f 3-9 lipo-

protein fraction, although the increment was less in the high density lipoprotein fraction.

Table 3 shows the tocopherol distribution in the lipoproteins following a lipid meal without supplementary tocopherol. One pint of ice cream, a test meal that produces lipemia without elevating serum tocopherol, was given to subject I. Blood specimens were obtained at 0, 2, 4, and 8 hours, and serum lipoprotein fractions isolated. The lipoprotein fractions were analyzed for tocopherol (EE reaction), fatty acid ester equivalents, cholesterol, and phospholipid. The lipid meal produced visible lipemia and a significant increase in the concentration of the fatty acid esters in the chylomicron and S_f 10-400 lipoprotein fractions, without altering the tocopherol content of these two fractions. The lipid and tocopherol concentrations of the S_f 3-9 and high density lipoprotein fractions were not altered by the lipid meal.

TABLE 3. COMPOSITION OF THE CHYLOMICRON AND THE S_f 10-400 LIPOPROTEIN FRACTIONS AFTER A LIPID MEAL (ICE CREAM) WITHOUT SUPPLEMENTARY TOCOPHEROL *

Fraction and Time in Hours	Tocopherol	Fatty Acid Ester	Ratio †
	μg./ml.	*meq./100 ml.*	
Chylomicron			
0	0.30	0.004	17.2
2	0.33	0.010	7.6
4	0.33	0.070	1.1
8	0.30	0.030	2.3
S_f 10-400 lipoproteins			
0	1.44	0.11	3.0
2	1.48	0.18	1.9
4	1.48	0.21	1.6
8	1.07	0.15	1.6

* Density gradient ultracentrifugal flotation procedure (5, 6).
† Tocopherol (μmole)/fatty acid ester (meq.).

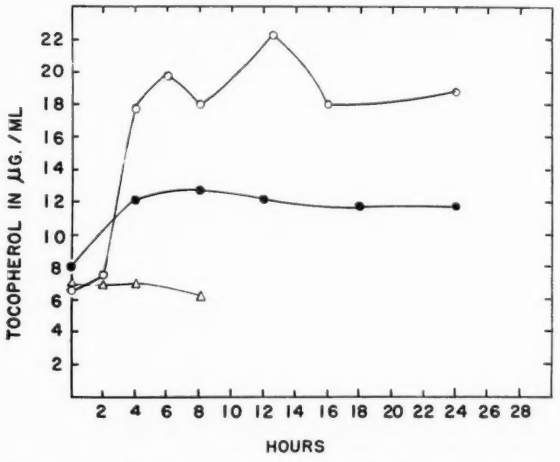


FIG. 1. The concentration of tocopherol (EE reaction) in serum after oral administration. Tocopherol, 2.0 g., administered to subject II with a lipid meal (o). Tocopherol, 600 mg., administered to subject I without a lipid meal (●). Lipid meal without supplementary tocopherol administered to subject I (Δ).

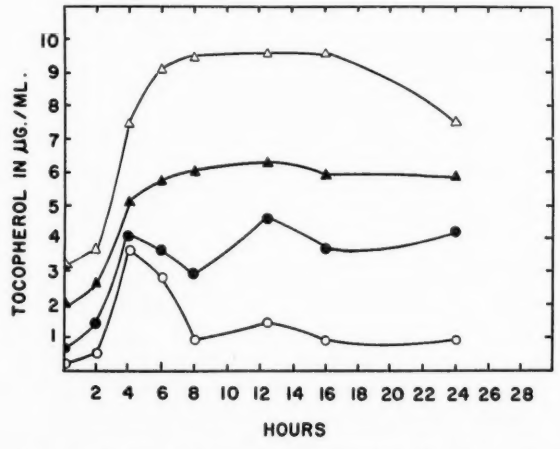


FIG. 2. The tocopherol content (EE reaction) in μg./ml. serum for lipoprotein fractions separated by density gradient flotation from subject II after the oral administration of 2.0 g. tocopherol with a lipid meal: chylomicrons (o), S_f 10-400 lipoproteins (●), S_f 3-9 lipoproteins (Δ), high density lipoproteins and other serum proteins (▲).

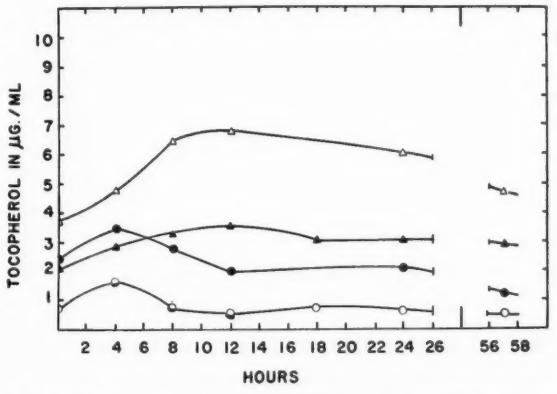


FIG. 3. The tocopherol content (EE reaction) in μg./ml. serum for lipoprotein fractions separated by density gradient flotation from subject I after the oral administration of 600 mg. tocopherol without a lipid meal: chylomicrons (o), S_f 10-400 lipoproteins (●), S_f 3-9 lipoproteins (Δ), high density lipoproteins and other serum proteins (▲).

Tocopherol Exchange Between Lipoproteins in Vitro. The tocopherol equilibration between low density and high density lipoprotein fractions was studied by tocopherol exchange *in vitro*. Blood was obtained from subject II before a test meal and 10 hours after the administration of 1.2 g. of tocopherol with a test meal. Duplicate low and high density lipoprotein fractions were separated by centrifugation at density 1.063. One lipoprotein fraction was used for a tocopherol exchange study; the other lipoprotein fraction was used for tocopherol analysis. A low density lipoprotein fraction from fasting serum was mixed with a high density lipoprotein fraction from the tocopherol-rich serum (Mixture A), and a low density lipoprotein fraction from the tocopherol-rich serum was mixed with a high density lipoprotein fraction from fasting serum (Mixture B). These lipoprotein mixtures were equilibrated at 37°C under nitrogen by agitation for 6 hours. Lipoprotein fractions were then reisolated by centrifugation at density 1.063 and analyzed for tocopherol. The results are summarized in Table 4. Tocopherol exchanged between the low and high density lipoprotein fractions. The final tocopherol distributions, 57 to 61 per cent in low density lipoproteins and 39 to 43 per cent in high density lipoproteins, were similar for the two mixtures. Furthermore, the percentage distribution of tocopherol obtained by equilibration *in vitro* was similar to the tocopherol distribution found in serum.

DISCUSSION

Essentially all serum tocopherol is found in the serum lipoprotein fractions. The small amount of apparent tocopherol found in the other serum proteins (density > 1.21) may be due to either vitamin A alcohol (6), which reacts in the analytical procedures, or to the incomplete separation of high density lipoproteins during the isolation procedure (4). If tocopherol were associated with other serum proteins, their tocopherol content should increase with increased levels of serum tocopherol; but this did not occur. Although albumin has been shown to combine with tocopherol *in vitro* (17), it does not appear to participate in tocopherol transport *in vivo*. Other workers did not detect radioactivity in the serum albumin fraction isolated from rats fed C^{14}-tocopherol (18).

In the fasting state the low density lipoproteins, principally the S_f 3-9, contain a major part of the serum tocopherol. This observation is in contradiction to an earlier observation (3) that the high density lipoproteins are the principal carriers of tocopherol. Independent studies have shown that elevated tocopherol levels and elevated low density lipoprotein levels occur in hypercholesterolemic disease states such as myxedema, idiopathic hypercholesterolemia, diabetes mellitus, xanthomatosis, coronary occlusion, cerebral thrombosis, and hypertensive cardiovascular disease (16, 19, 20, 21). During pregnancy there is a parallel rise in tocopherol (22) and low density lipoprotein levels (23), which reaches a peak at the third trimester.

Following oral administration of tocopherol with a lipid meal, the concentration of tocopherol in the chylomicron and S_f 10-400 lipoprotein fraction reaches a maximum value in 3 to 4 hours, a time at which the lipid content of these two fractions is also at a maximum (24, 25, 26). In control experiments, the separate administration of either tocopherol or lipid produces an independent elevation of either tocopherol or lipid, respectively, in the chylomicron and S_f 10-400 lipoprotein fractions. Thus the initial elevation of tocopherol in these fractions after tocopherol absorption represents newly absorbed tocopherol and not the exchange of tocopherol between the different lipoproteins, whose relative concentration is changing as a result of simultaneous lipid absorption.

The first phase of tocopherol transport by the chylomicrons and S_f 10-400 lipoproteins is followed by a second phase in which newly absorbed tocopherol shows a prolonged elevation in the S_f 3-9 and high density lipoproteins. The prolonged elevation is a reflection of the long half life of these latter fractions (27) as contrasted to the very short half life of the chylomicron (28) and S_f 10-400 fractions (27). It is noteworthy that the tocopherol increment in the S_f 3-9 lipoprotein and the high density lipoprotein fractions reaches a maximum in 8 to 10 hours, a time when the chylomicron and S_f 10-400 lipoprotein fractions are approaching basal levels. It appears that tocopherol is transferred to the S_f 3-9 and high density lipoproteins from the chylomicrons and S_f 10-400 lipoproteins. Metabolic sequences for these reactions are not defined completely; however, the conversion of S_f 10-400 lipoproteins to S_f 3-9 lipoproteins *in vivo* has been demonstrated (27). High density lipoproteins and chylomicrons may also be related in their metabolism since these fractions have a similar protein moiety (29, 30) which is distinct immunologically from the low density lipoproteins (31). Tocopherol equilibration studies *in vitro* and the parallel course of tocopherol increments in the S_f 3-9 and high density lipoprotein fractions both suggest that tocopherol exchanges between low density and high density lipoproteins in a manner similar to cholesterol (28) and phospholipid (32). Thus the second phase of tocopherol transport, a to-

TABLE 4. TOCOPHEROL EXCHANGE, *In Vitro*, BETWEEN LOW DENSITY AND HIGH DENSITY LIPOPROTEIN FRACTIONS

Sample	Lipoprotein Tocopherol in μg./ml. and per cent (*italics*) of total recovered	
	Low Density D < 1.063	High Density D > 1.063
Serum fasting	5.9	3.5
	63	*37*
Serum 10 hours after test meal	17.2	7.0
	71	*29*
Mixture A		
Pre-equilibration	5.9	7.0
	*46**	*54**
Postequilibration (6 hours)	7.3	5.5
	57	*43*
Mixture B		
Pre-equilibration	17.2	3.5
	*83**	*17**
Postequilibration (6 hours)	12.5	8.0
	61	*39*

* Calculated for mixture.

Mixture A: Low density lipoproteins from fasting serum and high density lipoproteins from tocopherol-rich serum.

Mixture B: Low density lipoproteins from tocopherol-rich serum and high density lipoproteins from fasting serum.

copherol increment in the S_f 3-9 and high density lipoproteins, represents accumulation of tocopherol in the two major lipoprotein fractions of serum, together with exchange of tocopherol between these lipoproteins and a final tocopherol distribution similar to their total lipid content (33).

Total serum tocopherol shows an absorption increment of long duration because it enters into both the first and second phases of lipid transport by serum lipoproteins. Lipids such as triglycerides (25, 28) and vitamin A esters (6), which enter only the first phase of lipid transport, demonstrate an absorption increment of short duration. Triglycerides (34) and vitamin A esters (35) are both hydrolyzed after their initial concentration in the chylomicron and S_f 10-400 lipoprotein fractions and their hydrolysis products are subsequently not lipoprotein bound; whereas tocopherol is transferred to the S_f 3-9 and high density lipoproteins, which have a longer half life than do the chylomicrons and S_f 10-400 lipoproteins.

We are indebted to Mr. A. G. Worton, Warren-Teed Products Co., Columbus, Ohio, for samples of DL-α-tocopherol; Dr. Phillip L. Harris, Distillation Products Industries, Rochester, New York, for samples of D-α-tocopherol and β-carotene; Dr. G. Lakshminarayana for purified lecithin; and Dr. F. A. Kruger for his helpful suggestions.

REFERENCES

1. Ames, S. R., and H. A. Risley. *Ann. N.Y. Acad. Sci.* **52:** 149, 1949.
2. Ames, S. R. Unpublished observations cited by J. L. Oncley and F. R. N. Gurd in *Blood Cells and Plasma Proteins, Their State in Nature*, edited by J. L. Tullis, New York, Academic Press, Inc., 1953, p. 352.
3. Lewis, L. A., M. L. Quaife and I. H. Page. *Am. J. Physiol.* **178:** 221, 1954.
4. Havel, R. J., H. A. Eder and J. H. Bragdon. *J. Clin. Invest.* **34:** 1345, 1955.
5. Oncley, J. L., K. W. Walton and D. G. Cornwell. *J. Am. Chem. Soc.* **79:** 4666, 1957.
6. Krinsky, N. I., D. G. Cornwell and J. L. Oncley. *Arch. Biochem. Biophys.* **73:** 233, 1958.
7. Rosenkrantz, H. *J. Biol. Chem.* **224:** 165, 1957.
8. Quaife, M. L., N. S. Scrimshaw and O. H. Lowry. *J. Biol. Chem.* **180:** 1229, 1949.
9. Cuthbertson, W. F. J., R. R. Ridgeway and J. C. Drummond. *Biochem. J.* **34:** 34, 1940.
10. Farber, M., A. T. Milhorat and H. Rosenkrantz. *Proc. Soc. Exptl. Biol. Med.* **79:** 225, 1952.
11. Abell, L. L., B. B. Levy, B. B. Brodie, and F. C. Kendall. *J. Biol. Chem.* **195:** 357, 1952.
12. Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr. *J. Biol. Chem.* **207:** 1, 1954.
13. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6:** 158, 1953.
14. Scudi, J. V., and R. P. Buhs. *J. Biol. Chem.* **146:** 1, 1942.
15. Hove, E. L., and Z. Hove. *J. Biol. Chem.* **156:** 611, 1944.
16. Popper, H., A. Dubin, F. Steigmann, and F. P. Hesser. *J. Lab. Clin. Med.* **34:** 648, 1949.
17. Voth, O. L., and R. C. Miller. *Arch. Biochem. Biophys.* **77:** 191, 1958.
18. Sternberg, J., and E. Pascoe-Dawson. *Can. Med. Assoc. J.* **80:** 266, 1959.
19. Polonovski, J., and M. Jarrier. In *Third International Conference on Biochemical Problems of Lipids*, edited by R. Ruyssen, Brussels, 1956, p. 53.
20. Darby, W. J., M. E. Ferguson, R. H. Furman, J. M. Lemley, C. T. Ball, and G. R. Meneely. *Ann. N.Y. Acad. Sci.* **52:** 328, 1949.
21. Postel, S. *J. Clin. Invest.* **35:** 1345, 1956.
22. Darby, W. J., R. O. Cannon and M. M. Kaser. *Obstet. Gynecol. Survey* **3:** 704, 1948.
23. Oliver, M. F., and G. S. Boyd. *Clin. Sci.* **14:** 15, 1955.
24. Havel, R. J. *Am. J. Clin. Nutrition* **6:** 662, 1958.
25. Kruger, F. A., D. G. Cornwell, G. J. Hamwi, and J. B. Brown. *Am. J. Clin. Nutrition* **8:** 44, 1960.
26. Lakshminarayana, G., F. A. Kruger and D. G. Cornwell. *Federation Proc.* **18:** 269, 1959.

27. Gitlin, D., D. G. Cornwell, D. Nakasato, J. L. Oncley, W. L. Hughes, Jr., and C. A. Janeway. *J. Clin. Invest.* **37**: 172, 1958.

28. Fredrickson, D. S., D. L. McCollester, R. J. Havel, and K. Ono. In *Chemistry of Lipids as Related to Atherosclerosis*, edited by I. H. Page, Springfield, Ill., Charles C Thomas, 1958, p. 205.

29. Rodbell, M., and D. S. Fredrickson. *J. Biol. Chem.* **234**: 562, 1959.

30. Scanu, A., and I. H. Page. *J. Exptl. Med.* **109**: 239, 1959.

31. Briner, W. W., J. W. Riddle and D. G. Cornwell. *J. Exptl. Med.* **110**: 113, 1959.

32. Florsheim, W. H., and M. E. Morton. *J. Appl. Physiol.* **10**: 301, 1957.

33. Eder, H. A. *Am. J. Med.* **23**: 269, 1957.

34. Korn, E. D. In *Chemistry of Lipids as Related to Atherosclerosis*, edited by I. H. Page, Springfield, Ill., Charles C Thomas, 1958, p. 169.

35. Vanden Schrieck, H. G., and H. G. Kunkel. In *Third International Conference on Biochemical Problems of Lipids*, edited by R. Ruyssen, Brussels, 1956, p. 347.

Volume 1
Number 3

Phospholipid and triglyceride metabolism of excised rat diaphragm and the role of these lipids in fatty acid uptake and oxidation*

E. M. Neptune, Jr., H. C. Sudduth, D. R. Foreman, and F. J. Fash

Naval Medical Research Institute,
National Naval Medical Center,
Bethesda 14, Maryland

[Received for publication September 14, 1959]

SUMMARY

After a 48-hour fast, the phospholipid fatty acids of rat diaphragm are reduced much more than the triglyceride fatty acids. Phospholipid thus appears to contribute more to the labile fatty acids than triglyceride. Both long- and short-chain C^{14}-labeled fatty acids are incorporated into triglyceride but only the long-chain fatty acids are incorporated into phospholipid. After incubation with palmitate-1-C^{14} and transfer to substrate-free Ringer phosphate, triglyceride specific activity decreases but phospholipid specific activity increases. During incubation with palmitate-1-C^{14} the triglyceride specific activity rises, then remains constant. Phospholipid specific activity is initially the same as triglyceride but rises at a nearly linear rate. There is net synthesis of triglyceride but not of phospholipid. Combustion of long-chain fatty acids to CO_2 indicates the existence of an endogenous dilution pool. It is proposed that phospholipids and triglycerides participate in the oxidation of saturated fatty acids.

In a previous communication (1) labile fatty acid concentrations were measured in excised rat diaphragm. The fall in concentration of these fatty acids during a 4-hour incubation could, in theory, support nearly all of the respiration of this tissue. In the earlier study only total fatty acids were determined. The present studies were designed to investigate the contribution of the major lipid components, phospholipid and triglyceride, to the labile fatty acids and the interrelationship of these lipids.

MATERIAL AND METHODS

Incubation Techniques. Diaphragm muscle was obtained as described previously (2). In the transfer experiments additional diaphragm was obtained from donor rats and incubated in the transfer recipient flask; these were discarded just at the time of transfer. Zierler (3) has presented evidence that diaphragm is metabolically dependent on enzymes that leak out into the surrounding medium during *in vitro* incubation. This donor incubation was employed to minimize depression of metabolism upon transfer to another medium. The incubation medium was Ringer phosphate, calcium free, as described previously (2). The flasks were incubated in a Dubnoff shaking metabolic incubator at 37°C.

Extraction of Lipids. At the end of the experimental period the tissues were removed, blotted on filter paper, and ground in a porcelain mortar with 0.5 to 1.0 ml. of (3:1, v/v) alcohol-ether. The ether was freshly distilled each day from stock ether which contained a small quantity of hydroxylamine HCl. The muscle suspension was transferred to a 25-ml. volumetric flask with three or four washings of alcohol-ether to a total volume of 12 to 17 ml. The solution was brought to a boil three times in a boiling water bath, cooled, and made to 25 ml. with alcohol-ether. The contents of the flask were transferred to tubes and centrifuged at 3,000 rpm. for 2 minutes. One ml. of supernatant was transferred to an aluminum planchet for determination of radioactivity.

Triglyceride Determination. Three ml. was removed for determination of fatty acid-ester bonds by the hydroxylamine, ferric chloride method of Stern and Shapiro (4); the color from various concentrations of

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

diaphragm phospholipid was determined separately and used to correct the total ester value after phospholipids were determined. The final value, compared to a tripalmitin standard, was called the triglyceride fraction; triglyceride fatty acid values were obtained by assuming that 94 per cent of the triglyceride was composed of fatty acids.

Phospholipid Determination. The remaining 21 ml. was taken to dryness at 100°C under a stream of nitrogen; 1 ml. of hexane was added and the tube warmed briefly. Then 14 ml. of acetone and 0.1 ml. of saturated $MgCl_2$ in ethanol were added, mixed for 5 minutes, and cooled at 10°C for 14 to 18 hours or at −10°C for 2 hours. The mixture was centrifuged, the supernate decanted, and the precipitate washed with 10 ml. of acetone. Since the acetone wash never contained over 1 per cent of the radioactivity present in the original acetone supernate, the acetone wash was routinely discarded in the isotope experiments.

The acetone-$MgCl_2$-insoluble precipitate was dried under nitrogen and treated in one of two ways:

(*a*) To the precipitate, 1.5 ml. of 60 per cent KOH and 1.5 ml. of absolute ethanol were added. The tube was capped, heated for 2 hours at 100°C, cooled to room temperature, and 1.5 ml. of 18 N H_2SO_4 was added. After cooling, 10 ml. of *n*-hexane was added and the tube shaken on a mechanical shaker for one-half hour. After centrifugation, 4 ml. of the hexane was taken to dryness under N_2 and titrated as reported previously (1). The titrated fatty acids in this fraction are referred to as phospholipid fatty acids. One ml. of the hexane was dried on a planchet for determination of radioactivity.

(*b*) In certain phases of the work, the radioactivity of the intact phospholipid molecule was determined. The acetone-$MgCl_2$ precipitate was dried and cooled. Four ml. of chloroform was added and, after warming, the tube was shaken and centrifuged. One ml. was transferred to a planchet for determination of radioactivity. The remaining 3 ml. was dried, digested, acidified, and extracted as in (*a*).

A detailed comparison of the acetone-$MgCl_2$ technique with silicic acid chromatography has been published by Borgström (5). Although separation by silicic acid chromatography yielded better recovery of phospholipid than the acetone-$MgCl_2$ precipitation, Borgström was able to precipitate 98 to 99 per cent of the lipid phosphorus. In the present study, experiments demonstrated that 96.4 ± 0.63 per cent of the total phosphorus present in the lipid extract was precipitated by acetone-$MgCl_2$. Although Borgström found that about 4 per cent of labeled free fatty acid added to phospholipid was coprecipitated by acetone-$MgCl_2$, our observations with silicic acid chromatography of precipitated phospholipid indicate that there is no appreciable contamination of the phospholipid fraction by free fatty acid.

Radioactivity and Manometric Determinations. Radioactivity and manometric CO_2 determinations were performed as described previously (2) with the following exceptions: Initial and residual activities of the incubation media were determined by direct plating of 0.1 ml. of a 1:10 dilution of the aqueous solutions. Radioactivity of material in organic solvents was determined by drying 1 ml. of the solution in a planchet. "Triglyceride" radioactivity was determined on the acetone-$MgCl_2$ supernate. An aliquot of the acetone supernate was also dried, taken up in hexane, transferred and extracted with alkaline ethanol as described by Borgström (6), and the extractable radioactivity determined and tabulated as free fatty acids. Triglyceride values were corrected by subtracting radioactivity and free fatty acids. Recovery experiments using palmitic acid-1-C^{14} were in excellent agreement with those reported by Borgström.

Preparation of Albumin-Fatty Acid Complexes. The long-chain fatty acid-albumin complexes were prepared in the following manner: Both labeled and unlabeled fatty acids of a measured weight were dissolved in alcohol-ether, and aliquots were dried under nitrogen at 100°C when using saturated acids, or at 60°C when using unsaturated acids. The sodium salts were prepared by adding a slight excess of NaOH in ethanol, and subsequent drying under nitrogen. The albumin was dissolved in approximately 10 ml. of Ringer phosphate and warmed to 40°C on a water bath. To the dry fatty acid salt, 0.5 ml. of Ringer phosphate was added and the tubes heated under nitrogen to the temperature listed above. The cloudy mixture was then transferred to the albumin solution with two more 0.5 ml. washes of Ringer phosphate and the optically clear albumin-fatty acid solution made to volume.

The albumin in these experiments was bovine albumin fraction V or crystalline bovine albumin. The fatty acid content of these proteins was determined by digestion, extraction, and titration, and its value incorporated into all calculations of fatty acid concentrations.

RESULTS

Incorporation of Labeled Fatty Acid. The values for phospholipid and triglyceride fatty acids before and after a 4-hour incubation with diaphragm from fed rats are given in Table 1. Both phospholipids and

TABLE 1. PHOSPHOLIPID AND TRIGLYCERIDE FATTY ACIDS OF DIAPHRAGM BEFORE AND AFTER A 4-HOUR INCUBATION COMPARED WITH TOTAL FATTY ACIDS *

	Number of Experiments	A Phospholipid Fatty Acids	Number of Experiments	B Triglyceride Fatty Acids	A Plus B as Per Cent of Total Fatty Acids †
		mg./g. wet wt.		*mg./g. wet wt.*	
Fed					
Before	5	7.19	11	13.16	91
After	5	5.38	11	12.18	96
Difference		−1.81 ±0.41		−0.98 ±0.21	69
p		<0.01		<0.01	
Fasted 48 Hours					
Before	5	0.84	5	10.30	87
After	5	0.34	5	9.48	87
Difference		−0.50 ±0.126		−0.82 ±0.05	87
p		<.02		<0.001	

* One-half diaphragm was used as a control; the opposite half was incubated for 4 hours. Total volume of medium was 3.0 ml.

† Total fatty acids from figures published previously (1). Variations are estimated standard errors.

triglycerides decrease during the incubation but there is a greater decrease in the phospholipid fatty acids. A more striking demonstration of the lability of phospholipid fatty acids can be seen in diaphragm from rats fasted for 48 hours. The preincubation level of phospholipids is only one-tenth the value for fed rats, whereas triglyceride fatty acids decrease by only 22 per cent. After a 4-hour incubation, these tissues from fasted rats have a somewhat greater fall in triglyceride fatty acids than phospholipid fatty acids, but the total fall in fatty acids is much less than noted with tissues from fed rats.

These results are in general agreement with the total fatty acid studies reported earlier (1). It is apparent that the phospholipid plus triglyceride values do not represent 100 per cent of the total fatty acids. Whether this is due to technical difficulties or to other unidentified lipid components has not been determined.

In Table 2 the results of studies are listed in which muscle was incubated with palmitate-, laurate-, oleate-, octanoate-, butyrate-, or acetate-1-C^{14}. All of the acids studied are incorporated into triglyceride; the greater incorporation of the short-chain acids may in part be due to the much higher concentration of these acids in the incubation medium. Although there is measurable incorporation of long-chain fatty acids in phospholipid, there is no incorporation of acetate, butyrate, or octanoate.

The great chemical lability of the phospholipids compared to that of triglycerides suggests that the phospholipids undergo a more rapid degradation than triglycerides and perhaps play a more active metabolic role. Therefore a series of experiments were performed in the following manner: Hemidiaphragms were incubated with C^{14}-labeled palmitate. At the end of 2 hours, one hemidiaphragm of each pair was removed for determination of lipids and radioactivity; the other hemidiaphragm was transferred to a flask containing unlabeled palmitate and incubated for 2 more hours, and then removed for determination of lipids and radioactivity. The results are shown in Table 3. Although during the second incubation there is no significant change in the concentrations of phospholipid, there is a significant fall in phospholipid specific activity. This indicates that there is a greater loss of labeled than of unlabeled fatty acid. The fall in specific activity of triglyceride indicates that this fraction is also losing labeled fatty acid faster than unlabeled. This experiment does not exclude transfer of labeled fatty acid from triglyceride to phospholipid, or the reverse.

To examine the interrelationship between triglyceride and phospholipid further, another series of experiments was performed in much the same way except that the tissues were transferred to Ringer phosphate without added substrate. The results are shown in Table 3. Although the fall in triglyceride concentration is paralleled by a fall in specific activity, there is a

TABLE 2. INCORPORATION OF C^{14}-LABELED PALMITATE, LAURATE, OLEATE, OCTANOATE, BUTYRATE, AND ACETATE INTO TRIGLYCERIDE, PHOSPHOLIPID, AND FREE FATTY ACID OF RAT DIAPHRAGM *

Added Substrate	Uptake	Triglyceride	Phospholipid	Free Fatty Acid
	mμmole/g.	*mμmole/g.*	*mμmole/g.*	*mμmole/g.*
Palmitate (5)	876 ± 86	260 ± 43	81 ± 8.2	42 ± 5.9
Laurate (4)	7121 ± 831	2250 ± 454	592 ± 129	920 ± 165
Oleate (6)	861 ± 120	191 ± 40	34 ± 6.5	86 ± 18.4
Octanoate (3)	———	4435 ± 1150	0	497 ± 291
Butyrate (5)	33,100 ± 3,400	167 ± 87	0	290 ± 106
Acetate (4)	16,800 ± 1,056	230 ± 69	0	490 ± 132

* One-half diaphragm from fed rat was incubated for 2 hours. Total volume was 2.7 ml. Substrates were present in the following concentrations: palmitate 0.9×10^{-4}, laurate 1.5×10^{-4}, oleate 1.0×10^{-4}, octanoate 22.2×10^{-4}, butyrate 44.4×10^{-4}, acetate 44.4×10^{-4}M. Palmitate, laurate, and oleate were all added as the albumin complex (see text). Figures in parentheses indicate the number of experiments. Uptake was calculated by measuring the residual activity in the medium and subtracting this from the initial activity. The percentage of radioactivity taken up was multiplied by chemical concentration to derive the mμmole values.

TABLE 3. CHANGES IN CHEMICAL VALUES AND SPECIFIC ACTIVITY OF TRIGLYCERIDE AND PHOSPHOLIPID OF DIAPHRAGM INCUBATED IN LABELED FATTY ACIDS AND THEN TRANSFERRED TO A SECOND MEDIUM *

Incubation Medium		Phospholipid		Triglyceride	
		Concentration	Specific Activity	Concentration	Specific Activity
		mg./g. wet wt.	*cpm./mg. fatty acid*	*mg./g. wet wt.*	*cpm./mg. fatty acid*
A. 2 hours in C^{14}-palmitate, then transferred to unlabeled palmitate (6)	2 hours	3.18	1209	16.3	1020
	4 hours	2.80	1025	13.7	718
	pair difference	−0.38 ±0.69	−177 ±50.8	−2.6 ±0.48	−302 ±75.6
	p value	>0.6	<0.01	<0.01	<0.01
B. 2 hours in C^{14}-palmitate, then transferred to Ringer phosphate (12)	2 hours	5.02	3196	19.0	3570
	4 hours	2.00	10,296	14.6	1226
	pair difference	−3.02 ±0.51	+7100 ±2110	−4.4 ±1.02	−344 ±98.4
	p value	<0.01	<0.01	<0.01	<0.01

* Incubation medium was Ringer phosphate, calcium free, pH 7.4, total volume 2.7 ml. One-half diaphragm was removed at 2 hours and the other half transferred and incubated for 2 more hours. Initial concentrations and radioactivity: A. = 0.9×10^{-4}M and 18,000 cpm./flask; B. = 1.2×10^{-4}M and 62,500 cpm./flask. The number of experiments in each series is in parentheses. Variations are expressed as standard errors.

striking rise in phospholipid specific activity despite a fall in concentration. This nearly threefold increase in specific activity is compatible with the concept that phospholipid is enriched from other lipid sources. There is again a depletion of labeled fatty acids in the triglyceride component.[1]

[1] These experiments were suggested by LCDR H. D. Baldridge, MSC, USN.

To elucidate the lipid component into which exogenous fatty acid is incorporated, some experiments were performed in which the early incorporation of labeled palmitate into phospholipid and triglyceride was studied. One hemidiaphragm served as a zero time control, while the opposite hemidiaphragm was incubated for a given time period with labeled palmitate. The results of this experiment are portrayed in Fig-

TABLE 4. OXIDATION OF FATTY ACID AND GLYCEROL SUBSTRATES TO $C^{14}O_2$ BY RAT DIAPHRAGM (2-HOUR OBSERVATION) *

Added Substrate	Concentration	$C^{14}O_2$ / Substrate C^{14} × 100	Total CO_2	CO_2 from Substrate / total CO_2 × 100
	$10^{-4}M$		*μmole*	
Laurate (8)	0.9	18	5.07	9.5 ± 0.60
Laurate (8)	1.8	28	5.99	26.0 ± 0.60
Palmitate (8)	0.9	7	5.90	5.4 ± 0.32
Palmitate (8)	1.2	9	4.76	10.1 ± 0.90
Oleate (12)	1.0	5	5.80	3.1 ± 0.30
Glycerol (12)	11.1	3	5.30	5.1 ± 0.60

* Quarter diaphragms were incubated in Dixson-Keilin flasks for 2 hours in 2.7 ml. of Ringer phosphate, calcium free, pH 7.4, 37°C. Substrate concentrations are listed in the Table. Techniques and calculations have been described elsewhere (2). Variations are estimated standard errors. Figures in parentheses indicate the number of individual experiments.

ure 1. At 10 minutes the specific activity of triglyceride is not significantly different from that of phospholipid, but although the triglyceride specific activity remains constant over the next 60 minutes there is a progressive rise in the specific activity of phospholipid. The concentrations indicate that there is an early net synthesis of triglyceride but not of phospholipid.

The Conversion of Fatty Acids to CO_2. In a separate series of experiments the extent to which muscle converts labeled long-chain fatty acids to $C^{14}O_2$ was determined. The results with palmitate-, oleate-, and laurate-1-C^{14} are shown in Table 4. Data for glucose have been reported elsewhere (2) as well as data for butyrate and octanoate. Contrary to the results for glucose and short-chain fatty acids, the conversion of long-chain fatty acids to $C^{14}O_2$ constitutes only a small fraction of the total CO_2. The labile fatty acids of diaphragm (1) are almost all esterified. If these fatty acids represent a dilution pool for exogenous long-chain fatty acids, then exogenous fatty acids such as palmitate should also be esterified if they are truly mixing with this endogenous pool. As shown in Table 2, added palmitate is incorporated into both triglyceride and phospholipid, which are the two sources of the endogenous labile fatty acids. Of course it is also possible that the free fatty acids might constitute a direct dilution pool. Although these fatty acids are present in low concentration (1), they could dilute directly and significantly the added labeled fatty acid if they were turning over rapidly.

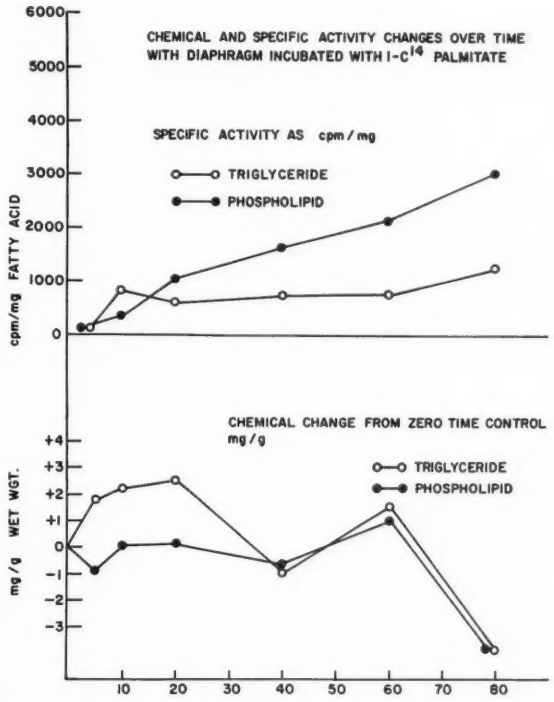


FIG. 1. One hemidiaphragm was used as a zero time control and the opposite hemidiaphragm was incubated for a given time period. The tissues were incubated in 2.7 ml. of Ringer phosphate. Palmitate-1-C^{14} was added in a concentration of 0.9 × 10^{-4}M. Initial activity was 29,000 cpm./flask. All values are the means of four separate experiments.

To test this pool concept, transfer experiments were performed in which muscle was incubated for 2 hours with labeled substrate, rinsed, and then transferred to vessels containing unlabeled substrate. The specific activities of CO_2 determined at different time intervals are shown in Figure 2. It is apparent that both glucose and octanoate show a definite fall in relative specific activity and the slopes of these curves are similar; the values for the specific activity of the CO_2 when diaphragm is incubated with palmitate do not fall. This indicates that the added labeled palmitate taken up by the tissue must be nearly completely equilibrated with the endogenous source of CO_2 over the time intervals studied and that entry of unlabeled fatty acid from the second medium is not rapidly diluting the endogenous pool. Furthermore, the constancy of the specific activity of the CO_2 after transfer to unlabeled substrate indicates that the dilution pool is large and this would not be compatible with the dilution pool existing in the form of free fatty acids; in the latter instance the specific activity of the CO_2 would rapidly decline after transfer to unlabeled substrate.

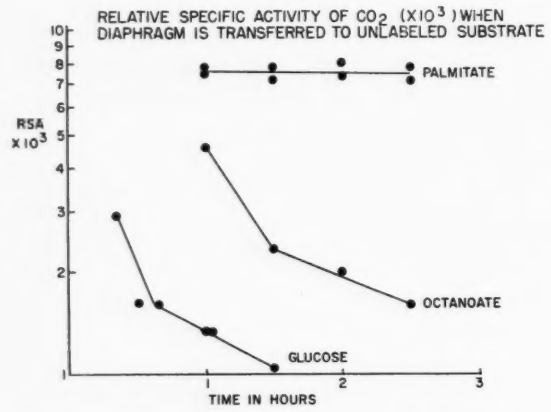


FIG. 2. Quarter diaphragms were incubated in Ringer phosphate, calcium free, pH 7.4 with one of the following substrates: glucose-U-C^{14} 82.6 $\times$ 10^{-4}M, octanoate-1-C^{14} 22.2 $\times$ 10^{-4}M, or palmitate-1-C^{14} 0.9 $\times$ 10^{-4}M for 1.5 hours. They were then transferred to Dixson-Keilin flasks containing the same amount of the same unlabeled substrates. Relative specific activity represents cpm./μmole CO_2/initial cpm.

DISCUSSION

It must be emphasized that in these initial studies phospholipids have been treated as a single group, whereas in actuality there are a variety of lipid compounds containing phosphorus, some of which undoubtedly have specific metabolic functions. The results of the present studies are sufficiently clear, however, to establish certain concepts.

It is evident from these studies that the endogenous labile fatty acids of rat diaphragm are contained in both phospholipid and triglyceride fractions. The phospholipid fraction is somewhat more labile during a 4-hour incubation and is strikingly reduced after a 48-hour fast.

Both long- and short-chain fatty acids labeled with C^{14} are incorporated into triglyceride, but of the acids studied only the long-chain acids, palmitate, oleate, and laurate, are incorporated into phospholipid. It is possible that the incorporation in triglyceride is mediated directly by lipase enzymes since the studies reported by Geyer *et al.* (7) show that striated rat muscle produces appreciable $C^{14}O_2$ when incubated with an emulsion containing carboxyl-labeled trilaurin, which presumably requires an active lipase. The failure of short-chain fatty acids to appear in the phospholipid fraction indicates a different mechanism which may depend, in part, on the difference in solubilities of short- and long-chain fatty acids. Bergström *et al.* (8) reported that in liver and intestine the incorporation of saturated acids C_{14}, C_{16}, and C_{18} into phospholipid was directly proportional to chain length. Hanahan and Blomstrand (9) found a much greater incorporation *in vivo* of palmitate than oleate into the lecithins of liver, intestine, and lymph. Stevens and Chaikoff (10) fed laurate-1-C^{14} and myristate-1-C^{14} and found 3 per cent of the laurate activity in carcass phospholipids as laurate and 19 per cent of myristate activity as myristate; most of the activity was found in the longer chain fatty acids and presumably represented elongation *in vivo* of the original 12 or 14 carbon fatty acids. Similarly, in our studies no incorporation of short-chain acids in phospholipid was observed. The monounsaturated acid, oleate, exhibited a lower proportion in phospholipid than laurate or palmitate.

In the study of the incorporation of palmitate with time, it appears that the specific activity of triglyceride remains at a constant level. There is an apparent net synthesis of triglyceride during the first hour, and during most of this period the specific activity is constant; this must mean that both labeled and unlabeled fatty acids are being incorporated in a constant proportion. The only obvious significant source of unlabeled fatty acid is phospholipid. The phospholipid specific activity values would indicate that labeled palmitate is incorporated more slowly in the very early part of the incubation, but that there is steady, almost linear, progressive enrichment.

The data presented do not clearly indicate the sequential pathway of incorporation of labeled palmitate entering the cell. It appears, however, that palmitate rapidly enters triglyceride and enters phospholipid more slowly; furthermore, fatty acid is apparently being transferred from phospholipid to triglyceride. If there is an active two-way shuttle of fatty acids between triglyceride and phospholipid, then palmitate may enter first into triglyceride and from there be more slowly incorporated into phospholipid. In experiments where diaphragm was transferred from labeled palmitate to buffered salt solution, it is possible to assume that low specific activity fatty acids are being removed from phospholipids with a net increase in their specific activity. The more tenable explanation, however, is that there is transfer to phospholipid of some high specific activity fraction of the triglyceride fatty acid.

The latter concept is supported by the experiments in which the tissues were transferred to unlabeled palmitate; in these there is no change in the amount of phospholipid, but there is a significant fall in specific activity; there is also a significant fall in the amount and specific activity of triglyceride. Therefore high specific activity fatty acids are leaving phospholipid and are also leaving triglyceride. It is most likely that these high specific activity fatty acids are being oxidized. It does not seem probable that, after transfer to unlabeled palmitate, fatty acids of high specific activity are removed from phospholipid, whereas after transfer to salt solution, fatty acids of low specific activity are preferentially removed. This, coupled with the observation that phospholipids fall more than triglycerides during an incubation or in starvation, would be compatible with the hypothesis that phospholipid fatty acids are transferred to oxidative enzyme systems for oxidative degradation.

The concept of transfer of saturated fatty acids from triglyceride to phospholipid was established in *in vivo* studies by Bergström *et al.* (8) when they fed triglyceride labeled with 1-C^{14} stearate. Although the specific mechanism was not elucidated by these workers, they suggested that transesterification might be involved. On the basis of the work of Stein and Shapiro (11) with liver preparations and also from the data of others (quoted by the authors) these investigators arrived at the "almost inescapable" conclusion that free fatty acid is first incorporated into triglyceride and then transferred to phospholipid. If this is the transfer mechanism in liver, intestine, and muscle, then this may represent a general role for phospholipids. The concept of such a role for phospholipid is not new and Artom has reviewed the historical background relating to this concept (12).

REFERENCES

1. Neptune, E. M., Jr., H. C. Sudduth and D. R. Foreman. *J. Biol. Chem.* **234**: 1659, 1959.
2. Neptune, E. M., Jr., H. C. Sudduth, F. J. Fash, and D. R. Foreman. *Am. J. Physiol.* **196**: 269, 1959.
3. Zierler, K. L. *Am. J. Physiol.* **185**: 12, 1956.
4. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6**: 158, 1953.
5. Borgström, B. *Acta Physiol. Scand.* **25**: 101, 1952.
6. Borgström, B. *Acta Physiol. Scand.* **25**: 111, 1952.
7. Geyer, R. P., L. W. Matthews and F. J. Stare. *J. Biol. Chem.* **180**: 1037, 1949.
8. Bergström, S., B. Borgström and M. Rottenberg. *Acta Physiol. Scand.* **25**: 120, 1952.
9. Hanahan, D. J., and R. Blomstrand. *J. Biol. Chem.* **222**: 677, 1956.
10. Stevens, B. P., and I. L. Chaikoff. *J. Biol. Chem.* **193**: 465, 1951.
11. Stein, Y., and B. Shapiro. *Am. J. Physiol.* **196**: 1238, 1959.
12. Artom, C. In *Phosphorus Metabolism*, edited by W. D. McElroy and B. Glass, Baltimore, Johns Hopkins Press, 1952, vol. 2, p. 235.

J. Lipid Research
April, 1960

Portal venous injection of insulin in the diabetic rat: time of induction of changes in hepatic lipogenesis, cholesterogenesis, and glycogenesis*

WALLACE R. WILLIAMS,† R. HILL,‡ and I. L. CHAIKOFF

Department of Physiology, University of California, Berkeley 4, California

[Received for publication December 3, 1959]

SUMMARY

The time of onset of changes in liver metabolism of the 14-hour-insulin-deprived, alloxan-diabetic rat following continuous portal vein infusion of insulin was studied. The earliest effect of insulin—on glycogen content of the liver and on the capacity of the liver slice to synthesize cholesterol from acetate—occurred between 10 and 30 minutes after the start of the infusion. The liver's capacity for incorporating acetate carbon into cholesterol rose dramatically between 30 and 60 minutes after the start of the hormone infusion, and returned to normal in 90 minutes. The significance of this biphasic response in hepatic cholesterogenesis to portal vein insulin infusion is discussed. Fatty acid synthesis was slowest to respond to insulin. An effect on hepatic lipogenesis was first detected between 60 and 90 minutes after injection, and the liver's capacity for incorporation of acetate-1-C^{14} into fatty acids continued to increase with duration of insulin administration up to 180 minutes.

The importance of duration of insulin action in evoking a response in hepatic lipogenesis of the diabetic rat was pointed out by Chernick and Chaikoff in 1950 (1). They demonstrated that pretreatment of diabetic rats with insulin for 1 day resulted in an increased utilization of glucose for fatty acid and CO_2 formation; insulin treatment for as short a period as 3 hours failed to induce this increased glucose utilization. The time element in insulin action on liver was later studied in greater detail by Hastings and his associates. The earliest effect observed by these workers occurred in 6 hours in chronic diabetic rats (2), and in 4 hours in 14-hour-insulin-deprived diabetic rats (3). In all the studies cited above, insulin was administered by a subcutaneous or by a nonportal, intravenous route. Since insulin produced by the pancreas is secreted directly into the portal circulation, we raised the question whether the reported values for the time element might, in some part, be an artifact of the peripheral route of insulin administration. For this reason we have investigated the time of onset of hepatic insulin action in diabetic rats in which the hormone was injected directly into the portal vein. When this route of administration is used, not only does the hormone enter the liver first, but also all of it reaches the liver.

* Aided by a grant from the United States Public Health Service.

† Public Health Service Research Fellow of the National Cancer Institute.

‡ Fellow of the American Heart Association.

EXPERIMENTAL

Male Long-Evans rats weighing from 225 to 275 g. were made diabetic by intravenous injections of alloxan-monohydrate (Eastman). Every day, each diabetic rat used in this study ingested about 30 g. of a stock diet (Diablo Labration), drank from 75 to 200 ml. of water, and excreted from 75 to 200 ml. of urine containing about 2 to 4 g. of glucose. Their blood sugars in the fed state ranged from 325 to 700 mg. per 100 ml. (Table 1).

At least 3 weeks were allowed to elapse after the alloxan injection before the diabetic rats were treated with insulin as described by Spiro and Hastings (4). On the first day each rat was injected subcutaneously with 10 units of protamine-zinc insulin per 100 g. body weight; on the second day, with 5 units per 100 g. body weight; on the third day, with 2 units per 100 g. body weight; and for the next 2 to 3 weeks, with 1 unit per 100 g. body weight. During the 2 days preceding the experiment, each rat was injected, in addition, with 1 unit of unmodified crystalline insulin (glucagon-free) 3 times daily.

The test diabetic rats had been deprived of insulin for exactly 14 hours at the time the cannulae were inserted into their portal veins. These test rats were first given, via the cannulae, a priming dose of 25 units of unmodified crystalline insulin (glucagon-free), followed immediately by infusion of a solution containing, per ml., 320 mg. of glucose and 5 units of unmodified insulin. Infusion was at the rate of 2.4 ml. per hour and lasted from 10 minutes to 3 hours.

The control rats were infused by portal vein with glucose only, from 10 minutes to 3 hours. The time of injection was adjusted so that, when the infusion was completed, the control rats had been deprived of insulin for exactly 14 hours. Thus the hepatic metabolism of each test rat was compared with that of a control which had been deprived of insulin for exactly 14 hours and which, in addition, had received the glucose infusion intraportally for the same period as had its corresponding test rat.

Approximately one-half hour before the start of the portal vein infusion, the rats were anesthetized with Nembutal® (10- and 30-minute-infused rats) or with ether (1- to 3-hour-infused rats). The duodenal portion of the small intestine was exteriorized, and small-bore polyethylene tubing (with an internal diameter of 0.023 inch reduced to proper diameter by stretching) was inserted deep into the inferior hepatico-duodenal vein so that the infusion fluid was delivered directly into the portal vein. The animals that were to be infused for 1 to 3 hours were placed in restraining cages. Each experiment consisted of at least 6 rats—from 3 to 5 test and from 2 to 4 control diabetic rats.

At the end of the infusion period the rats were rapidly killed by a blow on the head. Immediately thereafter blood was taken for determination of glucose, and a sample of liver was taken for determination of glycogen. The preparation of liver slices, the procedures used for their incubation with acetate-1-C^{14} and glucose evenly labeled with C^{14}, and the methods for determining C^{14}-fatty acids and C^{14}-cholesterol have been described elsewhere (5 to 8). Each incubation flask contained 2 μmoles of labeled acetate or 400 mg. per 100 ml. as labeled glucose. The amount of radioactivity in each flask was approximately 2×10^5 cpm.

Glycogen was isolated by the method of Sjögren *et al.* (9), and determined colorimetrically by the procedure of Mendel and Hoogland (10). In those cases in which the incorporation of the C^{14} of C^{14}-glucose into glycogen by liver slices was determined, the isolated glycogen was dialyzed to remove salts before it was plated on aluminum planchets for C^{14} counting.

RESULTS

Glycogenesis. The effects of infusion of glucose alone and of glucose plus insulin on blood glucose are recorded in Table 1. The effects of these treatments on liver glycogen are shown in this table and in Figure 1.

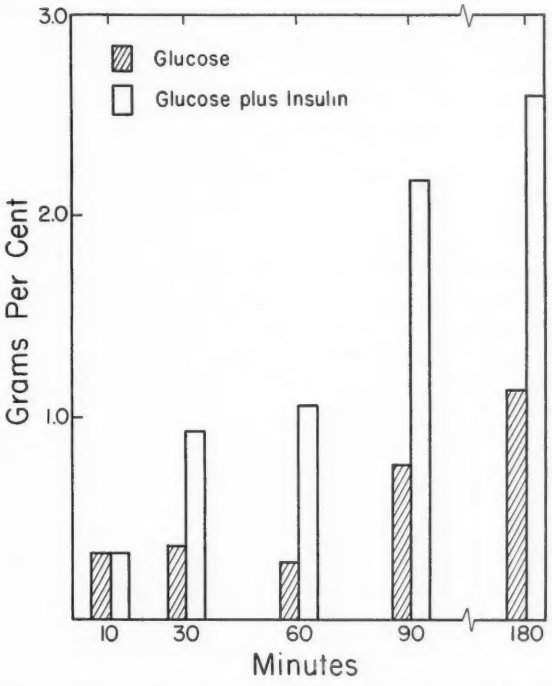


FIG. 1. Time course of the effects of continuous portal vein infusion of insulin on liver glycogen of intact diabetic rats. The control and test values for the 30-, 60-, 90-, and 180-minute experiments were significantly different at the 5% level as determined by the rank sum test (15).

The infusion of glucose alone (control diabetic rats) had no effect on the glycogen content of the liver for the first 60 minutes, but at later intervals of 90 and 180 minutes, the glycogen content of the livers of these control rats rose significantly.

No effect of insulin infusion for 10 minutes was detected, but by the time 30 minutes had elapsed, the administration of the hormone had increased the glycogen content of the liver.

The conversion of C^{14}- glucose to glycogen by slices prepared from the livers of insulin-treated and control rats was also studied. No effect of a 10-minute insulin infusion was noted. The infusion of the hormone for 30 minutes did, however, result in a fourfold increase in the liver's capacity for glycogenesis.

Lipogenesis. The continuous infusion of insulin for as long as 60 minutes failed to influence the liver's

TABLE 1. EFFECT OF INTRAPORTAL VEIN INFUSION OF INSULIN ON HEPATIC LIPOGENESIS, CHOLESTEROGENESIS, AND GLYCOGENESIS

Experiment	Number of Rats	Blood Sugar Before Infusion Treatment *		Infusion Treatment	Duration of Infusion	Liver Glycogen		cpm. of C^{14}-acetate Incorporated into				cpm. of Glucose-C^{14}† Recovered as				Blood Sugars at End of Infusion	
								Fatty Acid		Cholesterol		Fatty Acid		Glycogen			
		Range	Avg.			Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.
		mg./100 ml.	*mg./100 ml.*		*min.*	*per cent*	*per cent*										
1	5	386–511	446	Insulin + glucose	10	0.22–0.52	0.32	0–29	6	339–600	459			250–550	405	346–457	391
	4	380–467	431	Glucose	10	0.22–0.43	0.33	0–10	3	435–561	498			350–525	438	491–620	560
2	5	360–470	412	Insulin + glucose	30			0–17	3	958–1496	1122					190–222	206
	4	380–430	404	Glucose	30			0–21	9	525–991	729					510–690	588
3	5	383–450	414	Insulin + glucose	30	0.65–1.22	0.94							728–4527	1807	172–311	252
	4	376–428	398	Glucose	30	0.22–0.60	0.37							335–453	409	516–669	568
4	5	325–377	348	Insulin + glucose	60	0.45–1.51	1.06	0–147	56	1440–4378	2410	0–0	0			280–412	335
	4	336–531	431	Glucose	60	0.24–0.32	0.29	0–96	33	384–676	541	0–0	0			680–809	746
5	4	388–556	413	Insulin + glucose	90	1.35–3.20	2.19	190–525	316	308–1165	616	0–0	0			215–291	252
	2	462–570	516	Glucose	90	0.59–0.99	0.79	0–80	40	680–895	788	0–0	0			655–806	730
6	3	522–700	587	Insulin + glucose	180	2.24–2.86	2.61	1339–7215	3769	159–970	557	54–583	207			92–102	99
	3	442–630	505	Glucose	180	0.29–1.94	1.14	497–955	697	646–3460	1849	6–60	38			592–814	689

* These measurements were made during the 3-week (or longer) interval between the injection of the alloxan and the start of the insulin treatments as described by Spiro and Hastings (4).

† Evenly labeled.

capacity to synthesize fatty acids from acetate; an effect was, however, observed at 90 minutes (Table 1 and Fig. 2). An even greater delay was encountered in

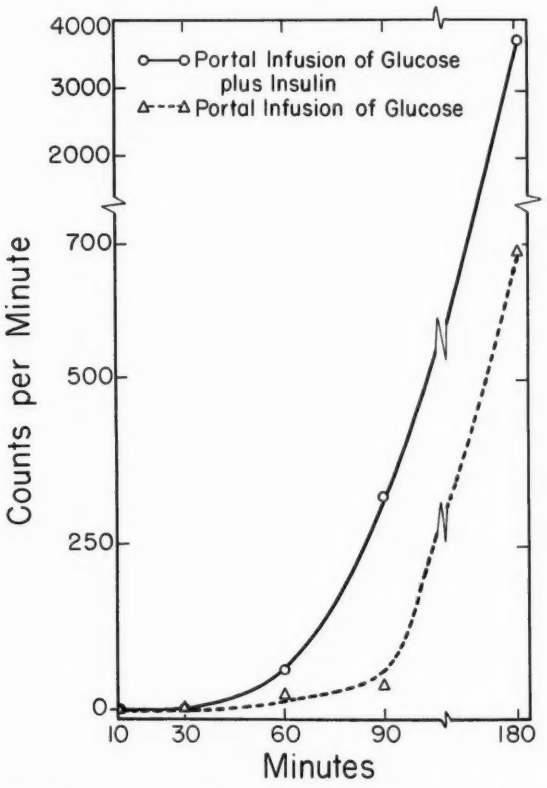


FIG. 2. Time course of the effects of continuous portal vein infusion of insulin on the capacity of liver slices of diabetic rats to convert the C^{14} of acetate-1-C^{14} to fatty acids. The control and test values differed significantly only in the 90-minute (at the 6% level) and in the 180-minute (at the 5% level) experiments, as judged by the rank sum test (15).

our attempt to obtain an action of insulin on fatty acid synthesis from C^{14}-glucose; a slight effect was detected in the 3-hour experiment, but not at 90 minutes.

A significant increase in lipogenesis from acetate was also observed at 3 hours in the control rats. But it should be noted that these 3-hour-glucose-infused rats started to received glucose when they had been deprived of insulin for only 11 hours.

Cholesterogenesis. The earliest effect of insulin on the capacity of the diabetic rat liver to synthesize cholesterol from acetate occurred at 30 minutes (Table 1 and Fig. 3). A biphasic response was noted. The augmented cholesterogenesis observed at 60 minutes was followed by a sharp decline in cholesterogenesis at 90 and 180 minutes. The prolonged infusion of glucose alone increased cholesterogenesis from acetate in control diabetic rats.

DISCUSSION

In order to study the time element in the induction of changes in the metabolism of the liver by portal infusion of insulin, we used the 14-hour-insulin-deprived, alloxan-diabetic rat. No effects on lipogenesis, cholesterogenesis, and glycogenesis were observed in 10-minute experiments. Effects of the hormone on glycogen formation were noted as early as 30 minutes after the start of the insulin infusion; this was shown not only in the levels of glycogen in the intact animal but also in the capacity of liver slices to incorporate C^{14}-glucose into glycogen *in vitro*. An unexpected result was the rapidity with which insulin affected cholesterol metabolism. Significant changes in the liver's capacity to synthesize this sterol occurred not later than 30 minutes. The greatest delay in the hormone's action was noted in the response of fatty acid synthesis from acetate, which occurred between 60 and 90 minutes after the start of the injection of insulin by way of the portal vein.

We reported earlier that the previous nutritional state of the rat determines the level of cholesterogenesis in the liver. Thus it was shown that fasting drastically reduces hepatic cholesterogenesis (11), and that liver slices prepared from normal rats fed a synthetic

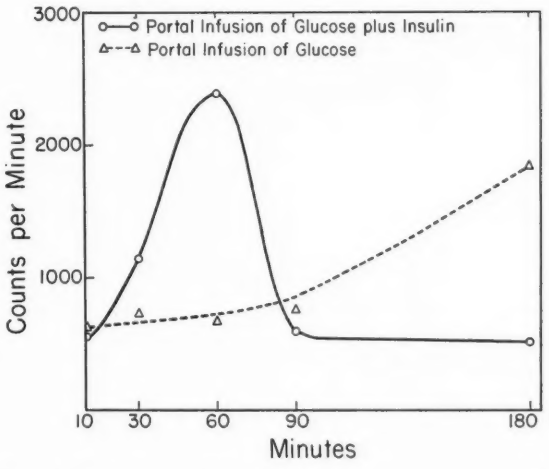


FIG. 3. Time course of the effects of continuous portal vein infusion of insulin on the capacity of diabetic rat liver slices to incorporate the C^{14} of acetate-1-C^{14} into cholesterol. Differences were significant at the 2% level in the 30-minute experiment (rank sum test) and at the 1% level in the 60-minute experiment. The values in the 180-minute experiment differed significantly at the 10% level.

diet containing 60 per cent glucose have a much greater capacity for incorporating acetate carbon into cholesterol than do slices from rats fed a 25 per cent glucose diet (12). A similar phenomenon was observed here—hepatic cholesterogenesis in the 14-hour-insulin-deprived, alloxan-diabetic rat is also sensitive to the availability of large amounts of carbohydrate.

An interesting aspect of the action of insulin on hepatic cholesterogenesis is the biphasic response shown in Figure 3. We have already seen that an augmented cholesterogenesis is associated with an increased glucose metabolism in the liver, and this accounts for the first effect of insulin (see 30 and 60 minutes in Fig. 3). The subsequent fall occurs at a time when the lipogenic effect of insulin has begun to take hold, and would appear to result from diversion of acetate toward fatty acid synthesis. A similar inverse relation between hepatic cholesterogenesis and lipogenesis was observed in experiments with normal rats fed high fat diets. In this case the depression in lipogenesis that followed fat feeding was accompanied by a rise in the extent of cholesterogenesis (13, 14).

Our thanks are due to Dr. W. R. Gaffey of the Department of Biostatistics in the School of Public Health for assistance in statistical treatment of the data.

REFERENCES

1. Chernick, S. S., and I. L. Chaikoff. *J. Biol. Chem.* **186:** 535, 1950.
2. Renold, A. E., A. B. Hastings, F. B. Nesbett, and J. Ashmore. *J. Biol. Chem.* **213:** 135, 1955.
3. Spiro, R. G., J. Ashmore and A. B. Hastings. *J. Biol. Chem.* **230:** 761, 1958.
4. Spiro, R. G., and A. B. Hastings. *J. Biol. Chem.* **230:** 751, 1958.
5. Katz, J., S. Abraham and N. Baker. *Anal. Chem.* **26:** 1503, 1954.
6. Baruch, H., and I. L. Chaikoff. *Endocrinology* **56:** 609, 1955.
7. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
8. Hotta, S., R. Hill and I. L. Chaikoff. *J. Biol. Chem.* **206:** 835, 1954.
9. Sjögren, B., T. Nordenskjöld, H. Holmgren, and J. Möllerström. *Arch. ges. Physiol. Pflüger's* **240:** 427, 1938.
10. Mendel, B., and P. L. Hoogland. *Lancet* **2:** 16, 1950.
11. Tomkins, G. M., and I. L. Chaikoff. *J. Biol. Chem.* **196:** 569, 1952.
12. Hill, R., J. W. Bauman and I. L. Chaikoff. *Endocrinology* **57:** 316, 1955.
13. Linazasoro, J. M., R. Hill, F. Chevallier, and I. L. Chaikoff. *J. Exptl. Med.* **107:** 813, 1958.
14. Hill, R., J. M. Linazasoro, F. Chevallier, and I. L. Chaikoff. *J. Biol. Chem.* **233:** 305, 1958.
15. Dixon, W. J., and F. J. Massey. *Introduction to Statistical Analysis,* 2d ed., New York, McGraw-Hill Book Co., 1957, p. 289.

Volume 1
Number 3

The transport and dynamic state of exogenous glycerol- and palmitic acid-labeled tripalmitin*

RAYMOND REISER, MARY C. WILLIAMS, and MARY F. SORRELS

Department of Biochemistry and Nutrition,
Texas Agricultural Experiment Station,
College Station, Texas

[Received for publication January 9, 1960]

SUMMARY

The radioactivities of the glycerol and fatty acid moieties of plasma, liver, and adipose tissue lipids were determined at intervals after the ingestion of glycerol- and palmitic acid-labeled tripalmitin by rats. The changes in the levels of activities of the glycerol and fatty acids, and in the ratios of those activities, demonstrated the following: Only about half of the glycerol is completely hydrolyzed from fatty acid during digestion and absorption. Plasma lecithin originating from dietary fat is synthesized extrahepatically. Plasma triglycerides are removed by the liver without change but are hydrolyzed before incorporation into adipose tissue. Liver lecithins originate from liver triglycerides. Triglycerides are metabolically more active than lecithins and the latter more active than cephalins. In the postabsorptive period, glycerol disappears from adipose tissue triglycerides more rapidly than do the fatty acids, indicating hydrolysis and renewal of glycerol. In the liver, on the other hand, glycerol and fatty acid disappear from both triglycerides and phospholipids at the same rates, indicating that they are removed as units.

In an earlier study from this laboratory, in which glycerol- and fatty acid-labeled triglycerides were used to investigate the mechanism of fat digestion (1), it was observed, though not reported, that after the deposition of the doubly labeled triglyceride in adipose tissue, the glycerol and fatty acid moieties disappeared at different rates. The present report is the result of an investigation on this phenomenon.

EXPERIMENTAL

Nineteen male rats, weighing approximately 200 g., were trained to eat a semisynthetic, fat-free ration[1] at 8:00 A.M. On the morning of the test, 200 mg. of tripalmitin, labeled in both the glycerol and fatty acid moieties, was fed with about 1 g. of the basal ration. The specific activity of the glycerol was 920,000 cpm. per mg. and that of the fatty acid was 125,000 cpm. per mg. The animals were subsequently fed the fat-free ration *ad libitum*.

Three animals were sacrificed by exsanguination through the aorta at 3, 6, 12, 24, and 72 hours after the test meal and four animals at 48 hours. The lipids were extracted from the plasma, the liver, and from the gross adipose tissue around the kidneys, testes, in the inguinal region, and under the skin. The plasma and liver lipids, about 30 mg. and 250 mg., respectively, isolated by the method of Folch *et al.* (2), were partitioned chromatographically on silicic acid columns (3).[2] The pooled adipose tissues from each animal were extracted with 10 volumes of chloroform in a Waring blendor and dried over silicic acid. The solvent was removed in a stream of nitrogen. The triglycerides from 100 mg. of the extracted adipose tissue lipid were isolated by column silicic acid chromatography (4).

The triglycerides of plasma, liver, and adipose tissues were saponified with 4 per cent alcoholic KOH. (In order to have samples of sufficient size for analyses, it was necessary to add unlabeled triglyceride as a carrier to the isolated and weighed plasma and liver triglycerides.) The fatty acids were extracted with petroleum ether (30°-60°C)[3] and dried. Twenty to 25 mg. samples were plated on "frosted" glass planchets,[4]

* Supported in part by Grant A-020 from the Robert A. Welch Foundation, Houston, Texas, and in part by Grant AT-(40-1)-2457 from the United States Atomic Energy Commission.

[1] Nutritional Biochemical Co., Cleveland, Ohio.

[2] Because of the small amounts available, the plasma of all the rats in each group was pooled.

[3] Skellysolve F.

[4] PL-F Full-Blasted Planchets, B and Z Enterprises, Columbus, Ohio.

TABLE 1. THE RADIOACTIVITIES OF THE FATTY ACID MOIETIES OF TISSUE LIPIDS AFTER THE INGESTION OF GLYCEROL- AND FATTY ACID-LABELED TRIPALMITIN *

Hours	Plasma †			Liver			Adipose
	TG ‡ *cpm./mg.*	Le ‡ *cpm./mg.*	Ce ‡ *cpm./mg.*	TG ‡ *cpm./mg.*	Le ‡ *cpm./mg.*	Ce ‡ *cpm./mg.*	TG ‡ *cpm./mg.*
3				495	462	270	4
				718	827	640	11
				1348	2047	1519	12
Average	2211	1105	569	854	1112	810	9
6				3715	3088	2513	588
				1649	1821	1337	247
				4144	2454	2685	411
Average	3931	2549	801	3169	2454	2178	415
12				2849	3137	2051	261
				1600	1558	1374	121
				1818	2336	1927	217
Average	152 §	2157	158 §	2089	2344	1784	200
24				1711	2385	2500	250
				1110	1565	1796	173
				2884	3377	2434	637
Average	2122	2871	1016	1902	2442	2243	353
48				77	700	729	196
				922	1340	1328	362
				919	1250	1323	455
				345	493	567	182
Average	352	591	227	566	946	987	299
72				219	257	321	333
				159	202	241	263
				233	279	290	385
Average	146	197	232	204	246	284	327

* Palmitic acid activity 125,000 cpm./mg.; glycerol activity 920,000 cpm./mg.; 200 mg. ingested in 1 g. of feed.

† Plasma of 3 animals pooled for analyses.

‡ TG = triglycerides; Le = lecithin; Ce = cephalins.

§ Unexplained dilution by unlabeled compound.

counted in a gas-flow counter to at least 5,000 counts, corrected for background and self-absorption, and their specific activities (cpm. per mg.) calculated. The glycerol was isolated from the saponification liquor as the tribenzoate derivative (5, 6). Infinitely thin platings of the derivative were made on "frosted" glass planchets, and the time for 5,000 counts determined with a gas-flow counter.

The liver and the plasma cephalins were saponified with 4 per cent alcoholic KOH, the fatty acids isolated and plated in the same manner as the triglyceride fatty acids. The plasma cephalins were diluted with unlabeled cephalin[5] to make the sample size suitable for analysis. Their glycerol activities were calculated from those of their methylene bismethone derivatives, prepared as previously described (7) from their glycerophosphate fractions.

The plasma and liver lecithin plus sphingomyelin fractions were differentially saponified by the method

[5] Isolated by silicic acid chromatography from rat liver lipids.

of Thannhauser *et al.* (8) in 0.25 N NaOH at 37°C for 6 days. The plasma lecithins were diluted with unlabeled lecithin[5] to make the sample size suitable for analysis. After decomposition of the soaps at a mild acid pH (methyl red), the mixtures were chilled and filtered through Celite pads on cold sintered glass funnels. The aqueous solutions contained the glycerophosphate of the lecithin. The C^{14} activities of the glycerophosphate were determined in the same manner as for the glycerophosphate of the cephalin fractions. The fatty acids and sphingomyelin were removed from the Celite pads with chloroform. The chloroform extracts were dried over anhydrous Na_2SO_4 and filtered. The dried and filtered chloroform solutions were slurried with 1 g. silicic acid in order to remove the sphingomyelin. The isolated fatty acids were dried and plated in the same manner as the fatty acids of the triglycerides and cephalins.

RESULTS AND DISCUSSION

Tables 1 and 2 present the relative specific activities of the fatty acid and glycerol moieties of liver and plasma cephalin, lecithin, and triglyceride, and of adipose tissue triglycerides.

Since the test fat was fed in a single meal, preceded and followed by the ingestion of a fat-free ration, the changes in the levels of the dietary fat into tissue lipids may be divided into two phases. The first phase is that during which the exogenous fat was incorporated into tissue lipids. This may be called the "absorption phase." The second phase is that during which the exogenous fat underwent changes in the tissues. The changes during the latter phase are a measure of the dynamic state of tissue lipids, and may be designated as the "postabsorption phase."

The changes in the activities of both fatty acids and glycerol in tissue lipids with respect to time show, with a few questionable exceptions, that the maximum levels of incorporation of dietary fatty acids and glycerol occurred at the sixth hour. This represents the absorption phase. The levels of activities of both fatty acid and glycerol in plasma and liver show that in the earlier hours after doubly labeled triglyceride ingestion, the exogenous fat is incorporated to the greatest extent into the triglycerides, less in the lecithins, and least in the cephalins. After the forty-eighth hour these relationships were reversed. These data lead to the conclusion that triglycerides are more dynamic, or "turn over" more rapidly, than lecithins, and that the cephalins are the most sluggish, though by no means inert. As will be discussed in more detail below, the data also indicate that the triglycerides are the precursors of lecithins. This may, at first sight, appear to be contrary to the mechanism of the syntheses of triglycerides and phospholipids as reported by Kennedy and Weiss (9, 10). The latter work, however, was on lipids synthesized *de novo*, while the present study describes the synthesis of lipids from exogenous triglycerides.

The radioactivities of the plasma and liver lecithin fatty acids were very similar at every period, which might mislead one to suspect the liver as the sole origin of the plasma phospholipid. However, the plasma lecithin glycerol activity was higher than that of the liver, at least during the first 24 hours. This apparent contradiction may be resolved by the concept that during, or shortly after, fat absorption plasma lecithin does not arise in the liver, but is formed mainly in the mucosa from exogenous fat. The low values of the labeled glycerol to fatty acid ratio in liver lecithin as compared to liver triglycerides, show that liver lecithin, on the other hand, arises mainly from triglycerides, as has been postulated by Harper *et al.* (11) as well as Stein and Shapiro (12).

The above discussion shows the danger of drawing conclusions with respect to precursor-product relationships of complex molecules, such as phospholipids, by following only one moiety. Furthermore, the dietary state of the animal and the experimental conditions must be taken into account. The injection of labeled P^{32} or C^{14}-acetate into a fasting animal, rather than an animal absorbing fat, may well result in quite different relationships between the levels of the label in lipids of various organs. Even greater differences should be expected if labeled lipids are being absorbed. For example, in studies with parenterally administered P^{32} (13, 14) and C^{14}-palmitic acid (15) in fasted liverless dogs, and C^{14}-acetate in pigs (4), the levels of labeled lipids indicated the hepatic origin of plasma phospholipid. On the other hand, oral administration of P^{32} resulted in a higher specific activity in the intestine and blood plasma than in the liver (16).[6] More recently, Zilversmit and Bollman, using P^{32} (17), found that the feeding of cream increased the amount of plasma phospholipid and concluded that after the feeding of fats, the intestine may contribute appreciably to plasma phosphatide.

The dissimilarity between glycerol activities of the plasma and liver cephalin, and the latter's lower levels,

[6] In a review of his work (*Phosphorus Metabolism*, Baltimore, Johns Hopkins Press, 1952, vol. 2, p. 223) Artom appears to have interpreted his data somewhat differently, concluding that labeled phosphorus in the liver and intestinal phospholipid reach about the same levels.

TABLE 2. THE RADIOACTIVITIES OF THE GLYCEROL MOIETIES OF TISSUE LIPIDS AFTER THE INGESTION OF GLYCEROL- AND FATTY ACID-LABELED TRIPALMITIN *

Hours	Plasma †			Liver			Adipose
	TG ‡ *cpm./mg.*	Le ‡ *cpm./mg.*	Ce ‡ *cpm./mg.*	TG ‡ *cpm./mg.*	Le ‡ *cpm./mg.*	Ce ‡ *cpm./mg.*	TG ‡ *cpm./mg.*
3				2224	459	347	0
				3217	923	638	0
				3882	2069	924	0
Average	8641	2833	1397	3107	1150	636	0
6				5343	1592	884	340
				1537	854	489	138
				5374	2550	1637	252
Average	12,400	4925	2364	4084	1665	1003	243
12				2886	2768	1569	259
				1449	1344	655	105
				1986	1655	1092	187
Average	247 §	4397	167 §	2107	1919	1105	183
24				1671	1578	1244	187
				1365	1183	822	130
				3325	2204	1639	245
Average	2395	3393	935	2120	1655	1235	187
48				51	559	781	186
				564	766	855	175
				310	553	674	102
				161	278	319	61
Average	95	512	162	272	539	657	131
72				77	183	227	81
				170	115	137	67
				84	135	151	102
Average	281	175	175	110	144	172	83

* Palmitic acid activity 125,000 cpm./mg.; glycerol activity 920,000 cpm./mg.; 200 mg. ingested in 1 g. of feed.

† Plasma of 3 animals pooled for analyses.

‡ TG = triglycerides; Le = lecithin; Ce = cephalins.

§ Unexplained dilution by unlabeled compound.

may be explained by the hypothesis that cephalin has a slower turnover rate than lecithin. (The irregularities in the response of the "cephalin" fraction are probably due to the complex nature of this fraction.) This was also demonstrated by the fact that both the glycerol and fatty acids of liver cephalin reached their maximum levels 12 hours or more after lecithin, and dropped off more slowly. Similar conclusions were reached by a study of differences in the activities and polyunsaturated fatty acid composition of cephalin in tissue lipids of pigs injected with labeled acetate (4).

Because of possible differences in the degrees of biological dilution, comparisons of the activities of the fatty acids or glycerol in lipids or tissues are undependable for some relationships, especially those of precursors and products. This difficulty may be circumvented by consideration of the ratios of the relative activities of the glycerol moieties to those of the fatty acids, a device which has been employed previously (7). Thus if this ratio in the ingested fat is considered as unity, any changes in the ratio are an indication of hydrolysis and resynthesis, and of the

TABLE 3. RELATIVE AMOUNTS OF GLYCEROL AS COMPARED TO FATTY ACIDS REMAINING IN TISSUE LIPIDS AFTER THE INGESTION OF GLYCEROL- AND FATTY ACID-LABELED TRIPALMITIN *

Hours	Plasma †			Liver			Adipose
	TG ‡	Le ‡	Ce ‡	TG ‡	Le ‡	Ce ‡	TG ‡
3				0.61	0.13	0.17	0
				0.61	0.15	0.14	0
				0.31	0.14	0.08	0
Average	0.53	0.35	0.33	0.51	0.14	0.13	0
6				0.19	0.07	0.05	0.08
				0.13	0.06	0.05	0.08
				0.18	0.14	0.08	0.08
Average	0.43	0.26	0.40	0.17	0.09	0.06	0.08
12				0.14	0.12	0.10	0.13
				0.12	0.12	0.06	0.12
				0.15	0.10	0.08	0.12
Average	0.22	0.28	0.14	0.14	0.11	0.08	0.12
24				0.13	0.09	0.07	0.10
				0.17	0.10	0.06	0.10
				0.16	0.09	0.09	0.05
Average	0.15	0.16	0.12	0.15	0.09	0.07	0.09
48				0.09	0.11	0.15	0.13
				0.08	0.08	0.09	0.07
				0.05	0.06	0.07	0.03
				0.06	0.08	0.08	0.05
Average	0.04	0.12	0.10	0.07	0.08	0.09	0.07
72				0.05	0.10	0.10	0.03
				0.14	0.08	0.07	0.04
				0.05	0.07	0.07	0.04
Average	0.26	0.12	0.10	0.08	0.08	0.08	0.03

* To allow for a much higher loss of glycerol as compared to fatty acid, the tripalmitin fed contained glycerol with a specific activity 7.4 times higher than the fatty acid. The relative loss of glycerol with respect to fatty acid is easier to understand if the relationship between the two is considered to be unity. Therefore the values in this table are equal to the expression: $\frac{\text{cpm./mg. glycerol}}{\text{cpm./mg. fatty acid} \times 7.4}$.

† Plasma of 3 animals pooled for analyses.

‡ TG = triglycerides; Le = lecithin; Ce = cephalins.

relative rates of disappearance of the two moieties. The differences in the ratios between the lipids may be used as a measure of product-precursor relations.

These ratios are given in Table 3. The values given here are not the actual ratios of glycerol to fatty acid activities but represent the changes from the dietary triglyceride. Thus the ratio of 0.5 in the liver triglycerides at the 3-hour period means that it incorporated only half as much of the labeled dietary glycerol as of fatty acid.

Even in the early stages of absorption of the test meal (Fig. 1), half as much of the labeled dietary glycerol as of fatty acids was incorporated into plasma triglycerides. This confirms the results of an earlier study on digestion (1) in which it was shown that up to 40 per cent of ingested glycerides are completely

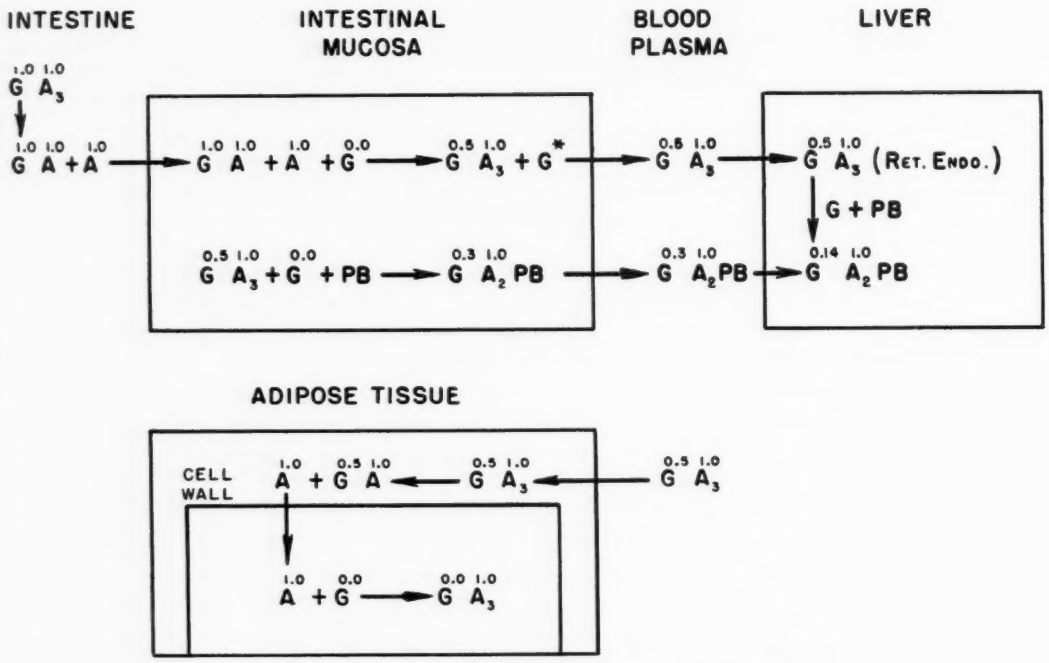


FIG. 1. Mechanism of absorption and transport of fat during the first 3 hours after ingestion. Superscripts represent activity of labeled glycerol as compared to labeled fatty acid. G = glycerol; GA_3 = triglyceride; GA = monoglyceride; GA_2PB = lecithin and cephalin; PB = phosphorylated choline or ethanolamine.

hydrolyzed during the digestion and absorption processes, and that the freed dietary glycerol is replaced by endogenous glycerol during glyceride resynthesis.

The relatively greater replacement of dietary glycerol in plasma phospholipids than in plasma triglycerides at the third hour (a ratio of 0.35 as compared to 0.53, Table 3 and Fig. 1) is a clear indication that the phospholipids are the product of more synthetic action than the triglyceride. Actually, the change in the ratio from 0.53 to 0.35 can be almost exactly accounted for by the stoichiometric reaction 2 GA_3 + GPB → 3 GA_2PB,[7] in which there is a one-third replacement of the glycerol. One can thus conclude that the triglyceride is the precursor of the phospholipid, probably in the mucosa, as has been postulated previously (7).

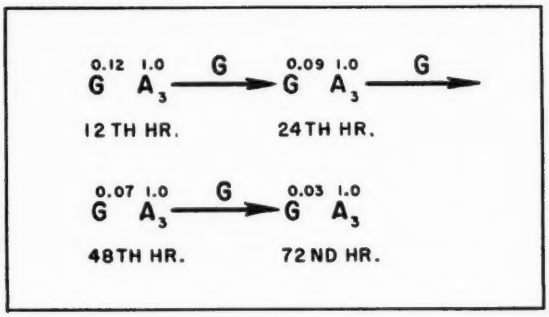
The relative amounts of active glycerol in the plasma and liver triglycerides are almost identical at the third hour, as shown by the ratios in Table 3, demonstrating retention of unmodified plasma triglyceride by liver reticulo-endothelium.

[7] G = glycerol; A = fatty acid; P = phosphate; B = nitrogenous base.

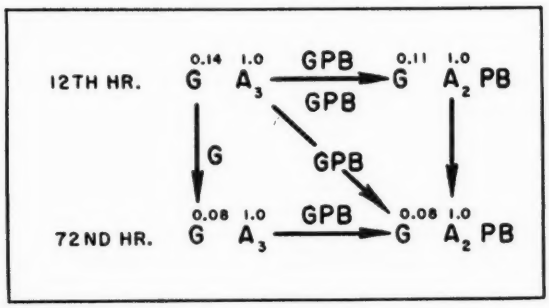
The much greater replacement of labeled glycerol in liver than in plasma phospholipids (both lecithin and cephalin) shows that liver phospholipids are not simply unmodified plasma phospholipids. In this respect they differ from the triglycerides, which are the same in both tissues. This again demonstrates that liver phospholipids must be a product of liver triglycerides. The degree of glycerol dilution from liver triglycerides to liver phospholipids (from 0.51 to 0.14) at the 3-hour period is greater than can be accounted for by the simple stoichiometric relationship outlined above for the plasma phospholipids. This may be interpreted to indicate a dynamic state of liver phospholipids in which they are rapidly being "turned over" with the labeled glycerol being replaced by unlabeled.

During the first 3 hours no measurable amount of labeled glycerol entered the fat depots (Table 2), although small amounts of fatty acids did (Table 1). During the second 6-hour period, glycerol actually entered the adipose tissue at a faster rate than the fatty acid, increasing the ratio to 0.12 at the twelfth hour, the end of the "absorption period."

A possible interpretation of this increase in the



ADIPOSE TISSUE



LIVER

FIG. 2. The dynamic state of tissue lipids during the postabsorptive state. Superscripts represent activity of labeled glycerol as compared to labeled fatty acid. G = glycerol; GA_3 = triglyceride; GA = monoglyceride; GA_2PB = lecithin and cephalin; PB = phosphorylated choline or ethanolamine.

glycerol:fatty acid ratio in the adipose tissue is that the plasma fat may be hydrolyzed to fatty acid and monoglyceride at, or in, the adipose tissue cell wall. The fatty acid may then enter the cell body more rapidly than the monoglyceride, and be rapidly resynthesized into triglyceride with ubiquitous unlabeled glycerophosphate. Thus the adipose tissue triglyceride would be labeled in the fatty acid only. Monoglyceride may enter more slowly, and, for that reason, toward the end of the absorptive period, would enter in relatively larger amounts than the fatty acid. This would cause a gradual increase in the labeled glycerol to labeled fatty acid ratio, as was obtained.

During the postabsorptive period (Fig. 2) the adipose tissue triglycerides maintained the largest percentage of their labeled fat of any lipid. Although the adipose tissue had the least loss of labeled fatty acid and glycerol, it should be noted that it was only in this tissue that the ratio of active glycerol to fatty acid changed significantly during the postabsorptive period up to 72 hours. It thus appears that triglycerides and phospholipids disappear from blood and liver as units, but that in adipose tissue, hydrolysis and resynthesis are part of the dynamic picture, with new glycerol replacing old during the process.

REFERENCES

1. Reiser, R., M. J. Bryson, M. J. Carr, and K. A. Kuiken. *J. Biol. Chem.* **194**: 131, 1952.
2. Folch, J., M. Lees and G. H. Sloan Stanley. *J. Biol. Chem.* **226**: 497, 1957.
3. Reiser, R., M. F. Sorrels and M. C. Williams. *Circulation Research* **7**: 833, 1959.
4. Reiser, R., M. C. Williams and M. F. Sorrels. *Arch. Biochem. Biophys.* **86**: 42, 1960.
5. Huntress, E. H., and S. P. Milliken. *Identification of Pure Organic Compounds.* New York, John Wiley & Sons, Inc., 1941, p. 479.
6. Miner, C. S., and N. N. Dalton. *Glycerol.* New York, Reinhold Publishing Corp., 1953, p. 174.
7. Reiser, R., and J. W. Dieckert. *Proc. Soc. Exptl. Biol. Med.* **87**: 622, 1954.
8. Thannhauser, S. J., J. Benotti and N. F. Boncoddo. *J. Biol. Chem.* **166**: 677, 1946.
9. Kennedy, E. P., and S. B. Weiss. *J. Am. Chem. Soc.* **77**: 250, 1955.
10. Kennedy, E. P., and S. B. Weiss. *J. Biol. Chem.* **222**: 193, 1956.
11. Harper, P. V., Jr., W. B. Neal, Jr. and G. R. Hlavacek. *Metabolism* **2**: 69, 1953.
12. Stein, Y., and B. Shapiro. *Am. J. Physiol.* **196**: 1238, 1959.
13. Fishler, M. C., C. Entenman, M. L. Montgomery, and I. L. Chaikoff. *J. Biol. Chem.* **150**: 47, 1943.
14. Entenman, C., I. L. Chaikoff and D. B. Zilversmit. *J. Biol. Chem.* **166**: 15, 1946.
15. Goldman, D. S., I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben. *J. Biol. Chem.* **184**: 727, 1950.
16. Artom, C., G. Sarzana, C. Perrier, M. Santangelo, and E. Serge. *Nature* **139**: 1105, 1937.
17. Zilversmit, D. B., and J. L. Bollman. *Arch. Biochem. Biophys.* **63**: 64, 1956.

Notes on Methodology

Application of a nomogram to gradient elution chromatography*

HUBER R. WARNER and WILLIAM E. M. LANDS
Department of Biological Chemistry,
The University of Michigan, Ann Arbor, Michigan

[Received for publication November 30, 1959]

► Gradient elution has been found to be an extremely useful procedure for separating mixtures on chromatographic columns since it provides an infinite number of solvent compositions, each with a different ability to elute the adsorbed materials. Constant volume gradient elution procedures were recently described by Hirsch and Ahrens (1), who showed the effectiveness of this procedure in separating plasma lipids. Although the stepwise change of solvent is useful, and even desirable in some cases (2), it is difficult to use trial-and-error procedures to choose the best solvent compositions. Gradient elution can be used initially to survey mixtures by column chromatography and to identify the solvent compositions at which various compounds are eluted from the column. Once the relationship between the extent of elution of the compound and solvent composition has been determined, it would be possible to predict the proper solvent mixtures for use later in stepwise elution procedures. In order to calculate conveniently the many different solvent compositions produced in constant volume gradient elution systems, a general equation has been derived and represented in the form of a nomogram (Fig. 1).

Assume (1) Two-solvent system, A and B.
(2) Constant volume mixing chamber.

Let X = volume fraction of A leaving mixing chamber.
X_0 = initial volume fraction of A in mixing chamber.
X_R = volume fraction of A in reservoir.
Q = amount of A in mixing chamber.
F = volume that has flowed through the mixing chamber.
V = volume of mixing chamber.

* Supported in part by a National Science Foundation Predoctoral Fellowship (H.R.W.) and Grant G-7647 from the National Science Foundation.

At any point in the elution, the change in Q will be

$$dQ = VdX$$

And $VdX = X_R dF - XdF = (X_R - X)dF$

Rearranging gives

$$\frac{dX}{X - X_R} = -\frac{dF}{V} \quad (1)$$

If X_R is constant, integration of (1) gives

$$\ln(X - X_R) = -\frac{F}{V} + C \quad (2)$$

When $F = 0, C = \ln(X_0 - X_R)$ (3)

Therefore,

$$\ln \frac{X - X_R}{(X_0 - X_R)} = -\frac{F}{V}$$

or

$$(X - X_R) = (X_0 - X_R)\, e^{-F/V} \quad (4)$$

A nomogram representing equation 4 can then be constructed, using the method described by Allcock and Jones (3).

Step 1. Two parallel lines are drawn 20 cm. apart to represent the loci of $(X_0 - X_R)$ and F/V. The locus of $(X_0 - X_R)$ is arbitrarily made 25 cm. long and is graduated linearly from 1.0 to 0.

Step 2. The following equation, $\Delta L_1 = 4e^{F/V} - 4.42$, where ΔL_1 is the linear distance in cm. between $F/V = 0.1$ and F/V, is used to calibrate the F/V locus. The point $F/V = 0.1$ should be directly opposite $(X_0 - X_R) = 1.0$.

Step 3. The third locus $(X - X_R)$ lies obliquely between the two parallel lines. A point lying on the $(X - X_R)$ locus is located by the intersection of two construction lines from corresponding values of F/V and $(X_0 - X_R)$ for any given value of $(X - X_R)$. Two such points determine the $(X - X_R)$ line.

Step 4. A line joining $(X_0 - X_R) = 1.0$ and $F/V = 0.1$ is graduated according to the following formula,

$$\Delta L_2 = \frac{125}{6.25 - \frac{1}{(X - X_R)}}$$

Projection lines, parallel to F/V and $(X_0 - X_R)$, are then drawn from these graduations to intersect the $(X - X_R)$ locus. These intersections represent the $(X - X_R)$ values used to calculate ΔL_2.

The separation of egg lecithin and lysolecithin by silicic acid chromatography provides an illustration of the usefulness of the nomogram shown in Figure 1.

The egg lecithin was purified by chromatography on alumina and the lysolecithin was prepared from this purified lecithin by the action of *Crotalus adamanteus* venom. A lipid sample containing 9 and 15 μmoles of lecithin and lysolecithin, respectively, was applied to a 5 g. silicic acid column (1 × 12 cm.) in 10 per cent methanol in chloroform. Either Mallinckrodt or Baker and Adams silicic acid (100 to 200 mesh) was used without any special activation. The column was washed with 16 ml. of 30 per cent methanol in chloroform, which removed a small amount of lecithin (about 0.05 μmoles of phosphorus per ml.). The gradient was started and the eluates (8 to 13 ml.) were collected in tubes on a fraction collector. The mixing chamber volume was 150 ml., while X_O and X_R for chloroform were 0.7 and 0.3, respectively. The solvent composition in each tube was then readily determined from the nomogram, knowing F/V for each tube. Straight lines connecting the point $X_O - X_R = 0.4$ with values of F/V intersect the value of $X - X_R$ related to F/V. In this example, $0.3 + (X - X_R)$ is the fraction of chloroform, and $0.7 - (X - X_R)$ is the fraction of methanol.

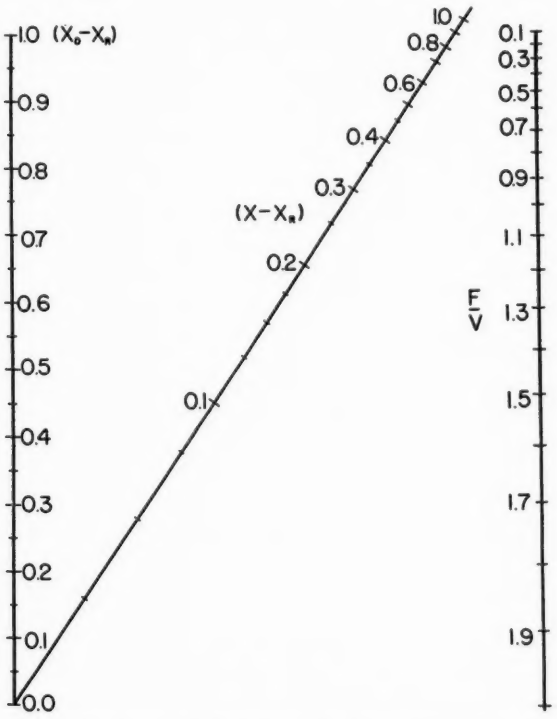


FIG. 1. Nomogram for determining the composition of solvent leaving the mixing chamber in constant volume gradient elution chromatography.

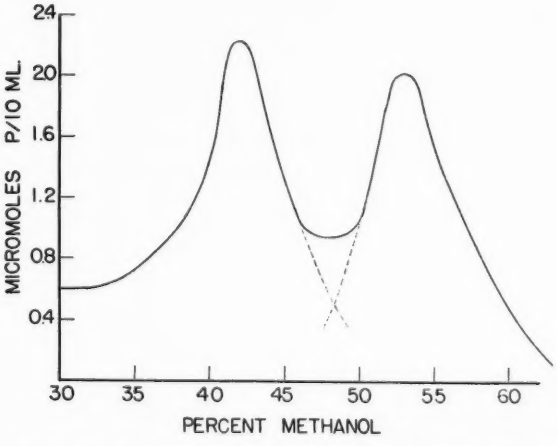


FIG. 2. Gradient elution of lecithin and lysolecithin from a silicic acid column by chloroform-methanol mixtures. The per cent methanol for each tube was calculated from the nomogram.

The total phosphorus concentration in each tube was determined after acid hydrolysis by the method of Fiske and Subbarow (4) and was plotted versus per cent methanol in Figure 2. The first peak is lecithin; the second is lysolecithin. The dashed lines represent the shape the peaks would assume if only one component were present.

The results from the gradient elution (Fig. 2) can now be used to develop a procedure for separating lecithin and lysolecithin using stepwise elution. Lecithin begins to move off the column very rapidly as the methanol concentration reaches 40 per cent, whereas lysolecithin is quite poorly eluted at this solvent composition. The lysolecithin, on the other hand, begins to move rapidly with 51 per cent methanol. A lipid mixture containing lecithin and lysolecithin may be resolved by adsorbing the lipid on the column and

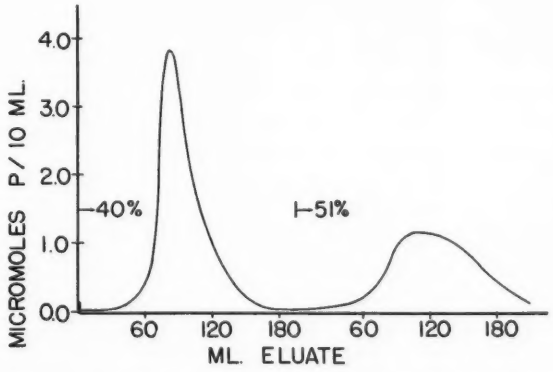


FIG. 3. Stepwise elution of lecithin and lysolecithin from a silicic acid column by chloroform-methanol mixtures.

washing with 40 per cent methanol in chloroform to elute the lecithin. After sufficient washing of the column with 40 per cent methanol, the lysolecithin may then be eluted with 51 per cent methanol in chloroform.

The stepwise elution of 16 μmoles of lecithin and 14 μmoles of lysolecithin is shown in Figure 3. In this experiment the lipids were added to a 5 g. silicic acid column in 10 per cent methanol in chloroform and eluted with 200 ml. of 40 per cent methanol and then 200 ml. of 51 per cent methanol.

In summary: A general equation describing the solvent composition flowing through the column in a constant volume gradient elution system has been developed and made into a nomogram. This nomogram is useful in quickly estimating solvent compositions and predicting procedures for separating components in a mixture by stepwise elution chromatography.

REFERENCES

1. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.
2. Hanahan, D. J., J. C. Dittmer and E. Warashina. *J. Biol. Chem.* **228**: 685, 1957.
3. Allcock, H. J., and J. R. Jones. *The Nomogram*, 4th ed., London, Sir Isaac Pitman & Sons, Ltd., 1950, pp. 47–58.
4. Fiske, C. H., and Y. Subbarow. *J. Biol. Chem.* **66**: 375, 1925.

Preparation of methyl esters*

NORMAN S. RADIN, AMIYA K. HAJRA, and YUKIO AKAHORI
Biochemistry Department, Northwestern University Medical School, Chicago 11, Illinois

[Received for publication December 15, 1959]

► The use of 2,2-dimethoxypropane (DMP) in preparation of methyl esters has been reported by Lorette and Brown (1). This reagent reacts very rapidly with water in the presence of strong acid to form methanol and acetone and thus can act as a water scavenger in esterification of acids with methanol. However, the possibility of direct methylation of acids has not been ruled out. We have modified the conditions described by these authors to speed the esterification and simplify the technique. The changes involve the use of aqueous hydrochloric acid as catalyst and relatively large amounts of reagents.

* Supported in part by United States Public Health Service Grant B-1179 of the National Institute of Neurological Diseases and Blindness.

A typical synthesis is carried out as follows: Up to 1 g. of stearic acid is placed in a 25 ml. Erlenmeyer flask or test tube, 2 ml. of methanol is added, then 0.2 ml. of concentrated hydrochloric acid, and then 5 ml. of DMP. After each addition of liquid the suspension is mixed, and then, when all reagents are added, the container is stoppered and left for 1 hour. If the acid is not completely dissolved on addition of the DMP, the flask is swirled until solution takes place, but the total reaction time is not extended.

The ester is isolated from the reaction mixture by transferring the solution to a larger flask (with the aid of 10 ml. of toluene for rinsing) and evaporating off the solvents under vacuum. The toluene reduces the tendency toward splashing during evaporation, and transfer is usually unnecessary when smaller amounts of acid are used. With large amounts of acid it is difficult to remove the last traces of toluene simply by evacuation. In this event, we redissolve the ester in petroleum ether (b.p. about 67°C) and re-evaporate under vacuum. The ester may also be isolated in the usual way, by addition of ether and water.

These conditions lead to quantitative yields with stearic acid, α-hydroxystearic acid, and other long-chain fatty acids. Lignoceric acid is rather insoluble in the reaction mixture, and we add 2 to 4 ml. of toluene after the DMP. Judging by the previous report (1) and by experiences of others with our method, we believe the method should be useful for most types of carboxylic acids. We have used the method, scaled accordingly, with samples of 15 mg. up to 50 g. of fatty acids, for preparative work and for gas chromatography.

Judging by the weight yields, there was complete esterification of the acids. Titration of the esters disclosed no free acid. Passage of the esters through Florisil,[1] using petroleum ether or petroleum ether-ethyl ether (2), yielded quantitative recovery of the esters; under these conditions free acids are adsorbed. A trace of yellow material forms during the esterification; this is adsorbed by Florisil and is presumably a polymer formed from the DMP. Heating the reaction mixture increases the yield of yellow material, and weight yields then run over 100 per cent. It is likely that the esterification is complete within a few minutes in our system, but we have not investigated shorter reaction times.

We redistilled the DMP[2] at atmospheric pressure (b.p. 80°-82°C), but subsequently discovered that Erley (3) recommends distillation at very low pres-

[1] Floridin Co., Tallahassee, Fla.
[2] Kindly furnished by Dow Chemical Co., Midland, Mich.

sure to avoid decomposition into methanol and 2-methoxypropene.

We are indebted to Dr. Maurice Rapport for bringing to our attention the potentialities of this interesting reagent.

REFERENCES

1. Lorette, N. B., and J. H. Brown, Jr. *J. Organic Chem.* **24**: 261, 1959.
2. Kishimoto, Y., and N. S. Radin. *J. Lipid Research* **1**: 72, 1959.
3. Erley, D. S. *Anal. Chem.* **29**: 1564, 1957.

Correction

In the article entitled "Studies on Blood Platelet Phospholipids," by Marcus *et al.*, in the January, 1960, issue, the words Dinitrophenyl Ethanolamine and Dinitrophenyl Serine should be substituted for Dinitrophenylhydrazine Ethanolamine and Dinitrophenylhydrazine Serine on lines 5, 6, and 7 in the left-hand column of page 184. It is to be noted that the abbreviation DNP as used elsewhere in the article refers to dinitrophenyl.

Notice of Meeting

The Fifth Meeting of the International Conference on Biochemical Problems of Lipids will take place in Marseilles, France, July 21-23, 1960. Subject: "The Enzymes of Lipid Metabolism." Sections: (1) Techniques, (2) Hydrolysis, (3) Oxidative Degradation, (4) Biosynthesis. For information write Prof. P. Desnuelle, Faculté des Sciences, Place Victor Hugo, Marseilles, France.

New Methods

► **New Methods is a listing of recent and pertinent references to analytical lipid methods published in other journals. It is compiled by Norman S. Radin, H. A. Newman, and David Kritchevsky. (References opened with No. 1 in the October, 1959, issue of the JOURNAL OF LIPID RESEARCH.)**

148. APPLICATION OF GAS-LIQUID CHROMATOGRAPHY TO ESSENTIAL-OIL ANALYSIS. Analytical Methods Committee. *Analyst* **84**: 690, 1959.

149. EVALUATION OF COMMERCIAL ALKYL ARYL SULFONATE DETERGENT AS COLUMN PACKINGS OR GAS CHROMATOGRAPHY. Desty, D. H., and C. L. A. Harbourn. *Anal. Chem.* **31**: 1965, 1959.

150. USE OF TWO OR MORE INTERNAL STANDARDS IN GAS CHROMATOGRAPHY. Lee, E. H., and G. D. Oliver. *Anal. Chem.* **31**: 1925, 1959.

151. SENSITIVITY OF CATHAROMETERS IN GAS CHROMATOGRAPHY AND THE THERMAL CONDUCTIVITY OF BINARY GAS MIXTURES. Littlewood, A. B. *Nature* **184**: 1631, 1959.

152. GAS-LIQUID RADIOCHROMATOGRAPHY. Popják, G., A. E. Lowe, D. Moore, L. Brown, and F. A. Smith. *Biochem. J.* **73**: 33P, 1959.

153. A METHOD OF EVALUATING SOLUBILITY PARAMETER FROM GAS CHROMATOGRAPHIC DATA. Takamiya, N., J. Kojima and S. Murai. *J. Chem. Soc. Japan, Ind. Chem. Sect.* **62**: 1371, 1959.

154. DESIGN AND USE OF A GAS CHROMATOGRAPHIC APPARATUS FOR THE ISOLATION OF ORGANIC REACTION PRODUCTS. *U. S. Govt. Research Repts.* **31**: 302, 1959. (PB 151 543., 75¢. Office of Technical Services, U. S. Department of Commerce, Washington 25, D.C.)

155. UNEXPECTED FORMATION OF VOLATILE COMPOUNDS ON GAS-LIQUID CHROMATOGRAPHY COLUMNS. Weurman, C., and J. Dhont. *Nature* **184**: 1480, 1959.

156. METHODS FOR THE ANALYSIS OF LIPIDS EXTRACTED FROM HUMAN ARTERIES AND OTHER TISSUES. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-van Houte, and C. M. van Gent. *Rec. trav. chim.* **78**: 794, 1959.

157. MICRO METHODS FOR THE DETERMINATION OF CHYLOMICRON COUNTS, FATTY ESTERS, LIPID PHOSPHORUS, AND CHOLESTEROL IN BLOOD SERUM. Clayton, M. M., P. A. Adams, G. B. Mahoney, S. W. Randall, and E. T. Schwartz. *Clin. Chem.* **5**: 426, 1959.

158. SÉPARATION DES LIPIDES PAR CHROMATOGRAPHIE SUR PAPIER IMPRÉGNÉ D'ACIDE SILICIQUE. II. POSSIBILITÉS ET LIMITES DE LA MÉTHODE. Cormier, M., P. Jouan and L. Girre. *Bull. soc. chim. biol.* **41**: 1037, 1959.

159. METABOLISM OF MYELIN LIPIDS: ESTIMATION AND SEPARATION OF BRAIN LIPIDS IN THE DEVELOPING RABBIT. Davison, A. N., and M. Wajda. *J. Neurochem.* **4**: 353, 1959.

160. A NEUTRAL REAGENT FOR THE ROUTINE DETERMINATION OF FAT IN SEPARATED MILK AND LOW-FAT PRODUCTS. Macdonald, F. J. *Analyst* **84**: 747, 1959.

161. CHROMATOGRAPHY OF LIPIDES. Noda, M. *Nippon Nôgeikagaku Kaishi* **33**: A17, 1959.

162. AN APPARATUS FOR DIRECT PHOTOMETRY OF LIPID ELECTROPHOREGRAMS. Visotsky, R. J., and V. F. Baumgart. *Voprosy Med. Khim.* **5**: 377, 1959.

163. A CONCAVE CONCENTRATION GRADIENT OF METHANOL IN CHLOROFORM EMPLOYED IN ELUTION OF LIPIDS FROM SILICIC ACID. Wren, J. J. *Nature* **184**: 816, 1959.

164. EXTRACTION METHODS AND AN INVESTIGATION OF DROSOPHILA LIPIDS. Wren, J. J., and H. K. Mitchell. *J. Biol. Chem.* **234**: 2823, 1959.

165. THE SEPARATION OF FREE 17-OH STEROIDS FROM STEROID CONJUGATES BY COLUMN CHROMATOGRAPHY. Brancaccio, A., B. D'Alessandro, F. Galdiero, and G. Jacono. *Ital. J. Biochem.* **8**: 139, 1959.

166. ESTIMATION OF 21 CARBON ATOM Δ-4-3-KETOSTEROIDS IN HUMAN URINE. Brancaccio, A., B. D'Alessandro, R. De Luca, and G. Jacono. *Ital. J. Biochem.* **8**: 143, 1959.

167. Zur Mikroskopischen Identitätsprüfung und zur Polymorphie der Sexualhormone. Brandstätter-Kuhnert, M., and A. Kofler. *Mikrochim. Acta*, 847 (1959).

168. A Method for the Estimation of Dehydroepiandrosterone in Urine. Fotherby, K. *Biochem. J.* **73:** 339, 1959.

169. Chromatographie sur Papier de Sécostéroïdes. Gounelle, J. C. *Bull. soc. chim. biol.* **41:** 1123, 1959.

170. Étude de la Bande de Vibration de Valence des Hydroxyles Stéroïdiques à l'État Associé. I. Fréquences. Henry, L., J. Weinmann and S. Weinmann. *Bull. soc. chim. biol.* **41:** 1175, 1959.

171. Pregnanetriol in Urine: Determination as a 17-Ketogenic Steroid. Morris, R. *Acta Endocrinol.* **32:** 596, 1959.

172. The Measurement of Pregnane-3α, 20α-Diol and Its Isomers in Human Plasma. Oertel, G. W., I. N. Beckmann and K. B. Eik-Nes. *Arch. Biochem. Biophys.* **86:** 148, 1960.

173. Die Anwendung der Wiederholten Entwicklung zur Trennung der Steroide auf dem Papier. Prusíková, M. *Experientia* **15:** 460, 1959.

174. Liquid Scintillation-Counting for (^{14}C) Steroids. Stitch, S. R. *Biochem. J.* **72:** 287, 1959.

175. Studies on the 'Differential' Hydrolysis of Steroid Conjugates in Urine. Paper Chromatographic and Colorimetric Determination of the Main Urinary Steroids Following Different Methods of Hydrolysis and Extraction. Teller, W., and W. Staib. *Acta Endocrinol.* **32:** 209, 1959.

176. Réactions Colorées de Stéroïdes. Wachsmuth, H., and L. Van Koeckhoven. *Anal. Chim. Acta* **22:** 41, 1960.

177. Elimination of Interfering Chromogens in the Zimmerman Reaction for Measuring 17-Ketosteroids. Ware, A. G., J. A. Demetriou, S. Notrica, R. Searcy, C. Walberg, and F. Cox. *Clin. Chem.* **5:** 479, 1959.

178. Recent Techniques of Separation and Determination of Bile Acids. Lambiotte, M. *Rev. intern. hépatol.* **7:** 521, 1959.

179. A Method for the Specific Differential Estimation of Cholic and Chenodeoxycholic Acids in Human Plasma. Reid, R. H. P., and G. S. Boyd. *Biochem. J.* **73:** 9P, 1959.

180. Rapid Semimicro Procedure for Estimating Free and Total Cholesterol. Courchaine, A. J., W. H. Miller and D. B. Stein, Jr. *Clin. Chem.* **5:** 609, 1959.

181. Studies of Cholesterol Methodology. Hollinger, N. F., E. Austin, D. Chandler, and R. K. Lansing. *Clin. Chem.* **5:** 458, 1959.

182. A Comparison of Methods for the Estimation of Serum Cholesterol and Values in Random Samples of Populations in the 55–64 Age Group. Morris, T. G. *J. Clin. Pathol.* **12:** 518, 1959.

183. Paper Chromatography of Steroids and Triterpenoids. II. Paper Chromatography of Sterols. Nishioka, I. *Yakugaku Zasshi* **79:** 1453, 1959.

184. Ozonolysis as a Method for Establishing the Position of Olefinic Linkages. Benton, F. L., A. A. Kiess and H. J. Harwood. *J. Am. Oil Chemists' Soc.* **36:** 457, 1959.

185. Paper Chromatography of 2,4-Dinitrophenylhydrazones of Saturated Aliphatic Aldehydes. Bhalerao, V. R., and F. A. Kummerow. *J. Am. Oil Chemists' Soc.* **36:** 461, 1959.

186. Quantitative Fatty Acid Analysis of Vegetable Oils by Gas-Liquid Chromatography. Craig, B. M., and N. L. Murty. *J. Am. Oil Chemists' Soc.* **36:** 549, 1959.

187. Determination of *CIS* Unsaturation in Oils by Near Infrared Spectroscopy. Fenton, A. J., Jr., and R. O. Crisler. *J. Am. Oil Chemists' Soc.* **36:** 620, 1959.

188. Column Chromatography of Lipides: Odd-Numbered Straight-Chain Fatty Acids of Menhaden Oil. Gellerman, J. L., and H. Schlenk. *Experientia* **15:** 387, 1959.

189. Gas Chromatographic Separation of Long-Chain Fatty Acid Methyl Esters on Polyvinyl Acetate. Hornstein, I., L. E. Elliott and P. F. Crowe. *Nature* **184:** 1710, 1959.

190. Chromatographic Separation of Fatty Acids Based on Chlorophenacyl Esters. Kibrick, A. C., and S. J. Skupp. *Anal. Chem.* **31:** 2057, 1959.

191. Note on a Method for the Extraction of Free Fatty Acids from Lipide Material. Mattick, L. R., and F. A. Lee. *Food Research* **24:** 451, 1959.

192. Derivatives of Ricinelaidic Acid. Their Infrared Spectra and Conformation of the Ricinelaidic Moiety. McCutchon, M. A., R. T. O'Connor, E. F. DuPré, L. A. Goldblatt, and W. G. Bickford. *J. Am. Oil Chemists' Soc.* **36:** 450, 1959.

193. Preparation and Properties of Methyl Arachidonate from Pork Liver. Privett, O. S., R. P. Weber and E. C. Nickell. *J. Am. Oil Chemists' Soc.* **36:** 443, 1959.

194. Mass Spectrometric Studies. II. Saturated Normal Long-Chain Esters of Ethanol and Higher Alcohols. Ryhage, R., and E. Stenhagen. *Arkiv Kemi* **14:** 483, 1959.

195. Micro-Determination of the Iodine-Value. Smits, P. *Rec. trav. chim.* **78:** 713, 1959.

196. A Simplified Technique for Analysis by Alkali Isomerization. White, H. B., Jr., and F. W. Quackenbush. *J. Am. Oil Chemists' Soc.* **36:** 653, 1959.

197. Separation of Glycerol Ethers by Gas-Liquid Chromatography. Blomstrand, R., and J. Gürtler. *Acta Chem. Scand.* **13:** 1466, 1959.

198. Méthode de Séparation Quantitative de Sphingomyélines. Applications à l'Étude du Renouvellement de la Sphingomyéline *in Vivo*. Bieth, R., G. Rebel and P. Mandel. *Bull. soc. chim. biol.* **41:** 1027, 1959.

199. Microdetermination of Phosphate in Range of 1 to 10 Micrograms. Hirata, A. A., and D. Appleman. *Anal. Chem.* **31:** 2097, 1959.

200. Chromatographic Separation on Alumina of the Total Cerebroside Plus Sulphatide from Rat Brain. Long, C., and D. A. Staples. *Biochem. J.* **73:** 7P, 1959.

201. Analysis of Human Plasma Phosphatides by Paper Chromatography. Marinetti, G. V., M. Albrecht, T. Ford, and E. Stotz. *Biochim. et Biophys. Acta* **36:** 4, 1959.

202. Effects of pH and Temperature on the Reduction of Periodates by Inositol. Sloane-Stanley, G. H., and P. A. Lock. *Biochem. J.* **73:** 8P, 1959.

203. A Gas Chromatographic Method for Sphingosine Assay. Sweeley, C. C. *Biochim. et Biophys. Acta* **36:** 268, 1959.

204. Paper Chromatography of Steroids and Triterpenoids. III. Paper Chromatography of Triterpenoids. Nishioka, I. *Yakugaku Zasshi* **79:** 1457, 1959.

205. Assay of Vitamin A Oils. Report on Three Collaborative Experiments. Brunius, E. *J. Assoc. Offic. Agr. Chemists* **42:** 657, 1959.

206. A Rapid Method for Determining the Constituent Components in Carotenoid Extracts from Leaf Tissue. Friend, J., and T. O. M. Nakayama. *Analyst* **84:** 654, 1959.

207. The Effect of Water on the Determination of Tocopherols. Hobson-Frohock, A. *Analyst* **84:** 567, 1959.

208. The Spectrophotometric Determination of α-Tocopherol. Lambertsen, G., and O. R. Braekkan. *Analyst* **84:** 706, 1959.

209. Electrophoretic Migration of Serum Lipoproteins in Starch Gel. Kutt, H., F. McDowell and J. H. Pert. *Proc. Soc. Exptl. Biol. Med.* **102:** 38, 1959.

210. A Modification of the Method for Prestaining Alpha and Beta Lipoproteins Separated by Paper Electrophoresis. Larkey, B. J., and J. S. Belko. *Clin. Chem.* **5:** 566, 1959.

211. Procedures for Determination of Lipoproteins by Electrophoresis on Paper. Vysotskaya, R. Y., and E. G. Livshits. *Lab. Delo*, 31 (1959).

INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4:** 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg.
gram	g.
milligram	mg.
microgram	μg.
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	$cm.^2$
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc.
microcurie	μc.
degrees of temperature	°C or °F

The expression mg.% should not be used, but should be written as mg. per 100 ml. For chemical nomenclature the JOURNAL will follow the conventions of *Chemical Abstracts*. The superscript ® should follow all trade names. When necessary for identification of other specific materials, use a footnote, including name and location of manufacturer.

References should appear in numerical order throughout the text. Bibliographic references must be typewritten, double-spaced, on a separate sheet, using the following style:

FOR JOURNAL ARTICLES: Author's last name, initials. *Name of journal* (abbreviated as in "List of Periodicals Abstracted," *Chemical Abstracts*, Supplementary Issue to Volume 50, 1956) volume number: page, year.

Example: Garn, S. M., A. B. Lewis and D. L. Polacheck. *Science* **128:** 1510, 1958.

FOR BOOKS: Author's last name, initials. *Title of Book.* City, Publishing House, year, page number.

Example: Adolph, E. F. *Physiology of Man in the Desert.* New York, Interscience Publishers, Inc., 1947, p. 420.

FOR ARTICLES IN BOOKS: Author's last name, initials. In *Title of Book*, initials and name of editor(s), City, Publishing House, year, volume, page number.

Example: Anker, H. S. In *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan, New York, Academic Press, Inc., 1957, vol. 4, p. 779.

(*Note:* In all references, reverse name and initials of *first-named author* only.)

Mention of "unpublished experiments," "personal communications," etc., should be made as footnotes and not included in the references. References to papers which have been *accepted* for publication but not yet printed, are cited in the style as other references, followed by the words "in press."

Send manuscripts to the JOURNAL OF LIPID RESEARCH, University of Tennessee, Memphis 3, Tenn.

INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4:** 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg.
gram	g.
milligram	mg.
microgram	μg.
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	$cm.^2$
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc.
microcurie	μc.
degrees of temperature	°C or °F

The expression mg.% should not be used, but should be written as mg. per 100 ml. For chemical nomenclature the JOURNAL will follow the conventions of *Chemical Abstracts*. The superscript ® should follow all trade names. When necessary for identification of other specific materials, use a footnote, including name and location of manufacturer.

References should appear in numerical order throughout the text. Bibliographic references must be typewritten, double-spaced, on a separate sheet, using the following style:

FOR JOURNAL ARTICLES: Author's last name, initials. *Name of journal* (abbreviated as in "List of Periodicals Abstracted," *Chemical Abstracts*, Supplementary Issue to Volume 50, 1956) volume number: page, year.
Example: Garn, S. M., A. B. Lewis and D. L. Polacheck. *Science* **128:** 1510, 1958.

FOR BOOKS: Author's last name, initials. *Title of Book.* City, Publishing House, year, page number.
Example: Adolph, E. F. *Physiology of Man in the Desert.* New York, Interscience Publishers, Inc., 1947, p. 420.

FOR ARTICLES IN BOOKS: Author's last name, initials. In *Title of Book*, initials and name of editor(s), City, Publishing House, year, volume, page number.
Example: Anker, H. S. In *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan, New York, Academic Press, Inc., 1957, vol. 4, p. 779.

(*Note:* In all references, reverse name and initials of *first-named author* only.)

Mention of "unpublished experiments," "personal communications," etc., should be made as footnotes and not included in the references. References to papers which have been *accepted* for publication but not yet printed, are cited in the style as other references, followed by the words "in press."

Send manuscripts to the JOURNAL OF LIPID RESEARCH, University of Tennessee, Memphis 3, Tenn.